

PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE

This application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 08/133,803 filed October 6, 1993.

FIELD OF THE INVENTION

5 The present invention relates generally to platelet-activating factor acetylhydrolase and more specifically to novel purified and isolated polynucleotides encoding human plasma platelet-activating factor acetylhydrolase, to the platelet-activating factor acetylhydrolase products encoded by the polynucleotides, to materials and methods for the recombinant production of platelet-activating factor acetylhydrolase products and to antibody substances specific for platelet-activating factor acetylhydrolase.

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BACKGROUND

15 Platelet-activating factor (PAF) is a biologically active phospholipid synthesized by various cell types. *In vivo* and at normal concentrations of 10⁻¹⁰ to 10⁻⁹ M, PAF activates target cells such as platelets and neutrophils by binding to specific G protein-coupled cell surface receptors [Venable *et al.*, *J. Lipid Res.*, 34: 691-701 (1993)]. PAF has the structure 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine. For optimal biological activity, the sn-1 position of the PAF glycerol backbone must be in an ether linkage with a fatty alcohol and the sn-3 position must have a phosphocholine head group.

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PAF functions in normal physiological processes (*e.g.*, inflammation, hemostasis and parturition) and is implicated in pathological inflammatory responses (*e.g.*, asthma, anaphylaxis, septic shock and arthritis) [Venable *et al.*, *supra*, and Lindsberg *et al.*, *Ann. Neurol.*, 30: 117-129 (1991)]. The likelihood of PAF involvement in pathological responses has prompted attempts to modulate the activity of PAF and the major focus of these attempts has been the development of antagonists of PAF activity which interfere with binding of PAF to cell surface receptors. See, for example, Heuer *et al.*, *Clin. Exp. Allergy*, 22: 980-983 (1992).

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30 The synthesis and secretion of PAF as well as its degradation and clearance appear to be tightly controlled. To the extent that pathological inflammatory actions of PAF result from a failure of PAF regulatory mechanisms

giving rise to excessive production, inappropriate production or lack of degradation, an alternative means of modulating the activity of PAF would involve mimicing or augmenting the natural process by which resolution of inflammation occurs. Macrophages [Stafforini *et al.*, *J. Biol. Chem.*, 265(17): 9682-9687 (1990)], hepatocytes and the human hepatoma cell line HepG2 [Satoh *et al.*, *J. Clin. Invest.*, 87: 476-481 (1991) and Tarbet *et al.*, *J. Biol. Chem.*, 266(25): 16667-16673 (1991)] have been reported to release an enzymatic activity, PAF acetylhydrolase (PAF-AH), that inactivates PAF. In addition to inactivating PAF, PAF-AH also inactivates 10 oxidatively fragmented phospholipids such as products of the arachidonic acid cascade that mediate inflammation. See, Stremler *et al.*, *J. Biol. Chem.*, 266(17): 11095-11103 (1991). The inactivation of PAF by PAF-AH occurs primarily by hydrolysis of the PAF *sn*-2 acetyl group and PAF-AH metabolizes oxidatively fragmented phospholipids by removing *sn*-2 acyl groups. Two types of PAF-AH have been identified: cytoplasmic forms found in a variety of cell types and tissues such as 15 endothelial cells and erythrocytes, and an extracellular form found in plasma and serum. Plasma PAF-AH does not hydrolyze intact phospholipids except for PAF and this substrate specificity allows the enzyme to circulate *in vivo* in a fully active state without adverse effects. The plasma PAF-AH appears to account for all of the PAF degradation in human blood *ex vivo* [Stafforini *et al.*, *J. Biol. Chem.*, 262(9): 4223-4230 (1987)].

While the cytoplasmic and plasma forms of PAF-AH appear to have identical substrate specificity, plasma PAF-AH has biochemical characteristics which distinguish it from cytoplasmic PAF-AH and from other characterized lipases. Specifically, plasma PAF-AH is associated with lipoprotein particles, is inhibited by 25 diisopropyl fluorophosphate, is not affected by calcium ions, is relatively insensitive to proteolysis, and has an apparent molecular weight of 43,000 daltons. See, Stafforini *et al.* (1987), *supra*. The same Stafforini *et al.* article describes a procedure for partial purification of PAF-AH from human plasma and the amino acid composition of the plasma material obtained by use of the procedure. Cytoplasmic 30 PAF-AH has been purified from erythrocytes as reported in Stafforini *et al.*, *J. Biol. Chem.*, 268(6): 3857-3865 (1993) and ten amino terminal residues of cytoplasmic PAF-AH are also described in the article. Hattori *et al.*, *J. Biol. Chem.*, 268(25):

-3-

18748-18753 (1993) describes the purification of cytoplasmic PAF-AH from bovine brain. Subsequent to filing of the parent application hereto the nucleotide sequence of bovine brain cytoplasmic PAF-AH was published in Hattori *et al.*, *J. Biol. Chem.*, 269(237): 23150-23155 (1994). To date no nucleotide sequence for the plasma form of PAF-AH has been published.

5 The recombinant production of PAF-AH would make possible the use of exogenous PAF-AH to mimic or augment normal processes of resolution of inflammation *in vivo*. The administration of PAF-AH would provide a physiological advantage over administration of PAF receptor antagonists because PAF-AH is a product normally found in plasma. Moreover, because PAF receptor antagonists which are structurally related to PAF inhibit native PAF-AH activity, the desirable metabolism of PAF and of oxidatively fragmented phospholipids is thereby prevented. Thus, the inhibition of PAF-AH activity by PAF receptor antagonists counteracts the competitive blockade of the PAF receptor by the antagonists. See, Stremler *et al.*,
10 *supra*. In addition, in locations of acute inflammation, for example, the release of
15 oxidants results in inactivation of the native PAF-AH enzyme in turn resulting in elevated local levels of PAF and PAF-like compounds which would compete with any exogenously administered PAF receptor antagonist for binding to the PAF receptor. In contrast, treatment with recombinant PAF-AH would augment endogenous PAF-AH activity and compensate for any inactivated endogenous enzyme.
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There thus exists a need in the art to identify and isolate polynucleotide sequences encoding human plasma PAF-AH, to develop materials and methods useful for the recombinant production of PAF-AH and to generate reagents for the detection of PAF-AH in plasma.

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SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated polynucleotides (*i.e.*, DNA and RNA both sense and antisense strands) encoding human plasma PAF-AH or enzymatically active fragments thereof. Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. The DNA sequence encoding PAF-AH that is set out in SEQ ID NO: 7 and DNA sequences which hybridize to the
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noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, are contemplated by the invention. Also contemplated by the invention are biological replicas (i.e., copies of isolated DNA sequences made *in vivo* or *in vitro*) of DNA sequences of the invention.

5 Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating PAF-AH sequences and especially vectors wherein DNA encoding PAF-AH is operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator are also provided.

According to another aspect of the invention, procarcytic or eucaryotic host cells are stably transformed with DNA sequences of the invention in a manner allowing the desired PAF-AH to be expressed therein. Host cells expressing PAF-AH products can serve a variety of useful purposes. Such cells constitute a valuable source of immunogen for the development of antibody substances specifically immunoreactive with PAF-AH. Host cells of the invention are conspicuously useful in methods for the large scale production of PAF-AH wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown by, for example, immunoaffinity purification.

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A non-immunological method contemplated by the invention for purifying PAF-AH from plasma includes the following steps: (a) isolating low density lipoprotein particles; (b) solubilizing said low density lipoprotein particles in a buffer comprising 10mM CHAPS to generate a first PAF-AH enzyme solution; (c) applying said first PAF-AH enzyme solution to a DEAE anion exchange column; (d) washing said DEAE anion exchange column using an approximately pH 7.5 buffer comprising 1mM CHAPS; (e) eluting PAF-AH enzyme from said DEAE anion exchange column in fractions using approximately pH 7.5 buffers comprising a gradient of 0 to 0.5 M NaCl; (f) pooling fractions eluted from said DEAE anion exchange column having PAF-AH enzymatic activity; (g) adjusting said pooled, active fractions from said DEAE anion exchange column to 10mM CHAPS to generate a second PAF-AH enzyme solution; (h) applying said second PAF-AH enzyme solution to a blue dye ligand affinity column; (i) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising 10mM CHAPS and a chaotropic salt;

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(j) applying the eluate from said blue dye ligand affinity column to a Cu ligand affinity column; (k) eluting PAF-AH enzyme from said Cu ligand affinity column using a buffer comprising 10mM CHAPS and imidazole; (l) subjecting the eluate from said Cu ligand affinity column to SDS-PAGE; and (m) isolating the approximately 44 kDa PAF-AH enzyme from the SDS-polyacrylamide gel. Preferably, the buffer of step (b) is 25 mM Tris-HCl, 10mM CHAPS, pH 7.5; the buffer of step (d) is 25 mM Tris-HCl, 1mM CHAPS; the column of step (h) is a Blue Sepharose Fast Flow column; the buffer of step (i) is 25mM Tris-HCl, 10mM CHAPS, 0.5M KSCN, pH 7.5; the column of step (j) is a Cu Chelating Sepharose column; and the buffer of step (k) is 25 mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, 50mM imidazole at a pH in a range of about pH 7.5-8.0.

A method contemplated by the invention for purifying enzymatically-active PAF-AH from *E. coli* producing PAF-AH includes the steps of: (a) preparing a centrifugation supernatant from lysed *E. coli* producing PAF-AH enzyme; (b) applying said centrifugation supernatant to a blue dye ligand affinity column; (c) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising 10mM CHAPS and a chaotropic salt; (d) applying said eluate from said blue dye ligand affinity column to a Cu ligand affinity column; and (e) eluting PAF-AH enzyme from said Cu ligand affinity column using a buffer comprising 10mM CHAPS and imidazole. Preferably, the column of step (b) is a Blue Sepharose Fast Flow column; the buffer of step (c) is 25mM Tris-HCl, 10mM CHAPS, 0.5M KSCN, pH 7.5; the column of step (d) is a Cu Chelating Sepharose column; and the buffer of step (e) is 25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, 100mM imidazole, pH 7.5.

Another method contemplated by the invention for purifying enzymatically-active PAF-AH from *E. coli* producing PAF-AH includes the steps of: (a) preparing a centrifugation supernatant from lysed *E. coli* producing PAF-AH enzyme; (b) diluting said centrifugation supernatant in a low pH buffer comprising 10mM CHAPS; (c) applying said diluted centrifugation supernatant to a cation exchange column equilibrated at about pH 7.5; (d) eluting PAF-AH enzyme from said cation exchange column using 1M salt; (e) raising the pH of said eluate from said cation exchange column and adjusting the salt concentration of said eluate to about

0.5M salt; (f) applying said adjusted eluate from said cation exchange column to a blue dye ligand affinity column; (g) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising about 2M to about 3M salt; and (h) dialyzing said eluate from said blue dye ligand affinity column using a buffer comprising about 0.1% Tween. Preferably, the buffer of step (b) is 25mM MES, 10mM CHAPS, 1mM EDTA, pH 4.9; the column of step (c) is an S sepharose column equilibrated in 25mM MES, 10mM CHAPS, 1mM EDTA, 50mM NaCl, pH 5.5; PAF-AH is eluted in step (d) using 1mM NaCl; the pH of the eluate in step (e) is adjusted to pH 7.5 using 2M Tris base; the column in step (f) is a sepharose column; the buffer in step (g) is 25mM Tris, 10mM CHAPS, 3M NaCl, 1mM EDTA, pH 7.5; and the buffer in step (h) is 25mM Tris, 0.5M NaCl, 0.1% Tween 80, pH 7.5.

PAF-AH products may be obtained as isolates from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving prokaryotic or eucaryotic host cells of the invention. PAF-AH products having part or all of the amino acid sequence set out in SEQ ID NO: 8 are contemplated. The use of mammalian host cells is expected to provide for such post-translational modifications (*e.g.*, myristylation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. PAF-AH products of the invention may be full length polypeptides, fragments or variants. Variants may comprise PAF-AH analogs wherein one or more of the specified (*i.e.*, naturally encoded) amino acids is deleted or replaced or wherein one or more nonspecified amino acids are added: (1) without loss of one or more of the enzymatic activities or immunological characteristics specific to PAF-AH; or (2) with specific disablement of a particular biological activity of PAF-AH. Proteins or other molecules that bind to PAF-AH may be used to modulate its activity.

Also comprehended by the present invention are antibody substances (*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and the like) and other binding proteins specific for PAF-AH. Specifically illustrating binding proteins of the invention are the monoclonal antibodies produced by hybridomas 90G11D and 90F2D which were

deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 on September 30, 1994 and were respectively assigned Accession Nos. HB 11724 and HB 11725. Proteins or other molecules (e.g., lipids or small molecules) which specifically bind to PAF-AH can be identified using PAF-AH isolated from plasma, recombinant PAF-AH, PAF-AH variants or cells expressing such products. Binding proteins are useful, in turn, in compositions for immunization as well as for purifying PAF-AH, and are useful for detection or quantification of PAF-AH in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for PAF-AH-specific antibody substances are also contemplated.

The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for PAF-AH makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding PAF-AH and specifying PAF-AH expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding allelic variants of PAF-AH, other structurally related proteins sharing one or more of the biochemical and/or immunological properties of PAF-AH, and non-human species proteins homologous to PAF-AH. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, e.g., Kapecchi, *Science*, 244: 1288-1292 (1989)], of rodents that fail to express a functional PAF-AH enzyme or that express a variant PAF-AH enzyme. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize PAF-AH. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the PAF-AH locus that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of PAF-AH by those cells which ordinarily express the same.

Administration of PAF-AH preparations of the invention to mammalian subjects, especially humans, for the purpose of ameliorating pathological inflammatory conditions is contemplated. Based on implication of the involvement of PAF in pathological inflammatory conditions, the administration of PAF-AH is indicated, for example, in treatment of asthma [Miwa *et al.*, *J. Clin. Invest.*, 82: 1983-1991 (1988); Hsieh *et al.*, *J. Allergy Clin. Immunol.*, 91: 650-657 (1993); and Yamashita *et al.*, *Allergy*, 49: 60-63 (1994)], anaphylaxis [Venable *et al.*, *supra*], shock [Venable *et al.*, *supra*], reperfusion injury and central nervous system ischemia [Lindsberg *et al.* (1991), *supra*], antigen-induced arthritis [Zarco *et al.*, *Clin. Exp. Immunol.*, 88: 318-323 (1992)], atherogenesis [Handley *et al.*, *Drug Dev. Res.*, 7: 361-375 (1986)], Crohn's disease [Denizot *et al.*, *Digestive Diseases and Sciences*, 37(3): 432-437 (1992)], ischemic bowel necrosis/necrotizing enterocolitis [Denizot *et al.*, *supra* and Caplan *et al.*, *Acta Paediatr., Suppl.* 396: 11-17 (1994)], ulcerative colitis (Denizot *et al.*, *supra*), ischemic stroke [Satoh *et al.*, *Stroke*, 23: 1090-1092 (1992)], ischemic brain injury [Lindsberg *et al.*, *Stroke*, 21: 1452-1457 (1990) and Lindsberg *et al.* (1991), *supra*], systemic lupus erythematosus [Matsuzaki *et al.*, *Clinica Chimica Acta*, 210: 139-144 (1992)], acute pancreatitis [Kald *et al.*, *Pancreas*, 8(4): 440-442 (1993)], septicemia (Kald *et al.*, *supra*), acute post streptococcal glomerulonephritis [Mezzano *et al.*, *J. Am. Soc. Nephrol.*, 4: 235-242 (1993)], pulmonary edema resulting from IL-2 therapy [Rabinovici *et al.*, *J. Clin. Invest.*, 89: 1669-1673 (1992)], allergic inflammation [Watanabe *et al.*, *Br. J. Pharmacol.*, 111: 123-130 (1994)], ischemic renal failure [Grino *et al.*, *Annals of Internal Medicine*, 121(5): 345-347 (1994); preterm labor [Hoffman *et al.*, *Am. J. Obstet. Gynecol.*, 162(2): 525-528 (1990) and Maki *et al.*, *Proc. Natl. Acad. Sci. USA*, 85: 728-732 (1988)]; and adult respiratory distress syndrome [Rabinovici *et al.*, *J. Appl. Physiol.*, 74(4): 1791-1802 (1993); Matsumoto *et al.*, *Clin. Exp. Pharmacol. Physiol.*, 19 509-515 (1992); and Rodriguez-Roisin *et al.*, *J. Clin. Invest.*, 93: 188-194 (1994)].

Animal models for many of the foregoing pathological conditions have been described in the art. For example, a mouse model for asthma, rhinitis, and eczema is described in Example 16 herein; a rabbit model for arthritis is described in Zarco *et al.*, *supra*; rat models for ischemic bowel necrosis/necrotizing

enterocolitis are described in Furukawa *et al.*, *Ped. Res.*, 34,(2): 237-241 (1993) and Caplan *et al.*, *supra*; a rabbit model for stroke is described in Lindsberg *et al.*, (1990), *supra*; a mouse model for lupus is described in Matsuzaki *et al.*, *supra*; a rat model for acute pancreatitis is described in Kald *et al.*, *supra*; a rat model for pulmonary edema resulting from IL-2 therapy is described in Rabinovici *et al.*, *supra*; a rat model of allergic inflammation is described in Watanabe *et al.*, *supra*); a canine model of renal allograft is described in Watson *et al.*, *Transplantation*, 56(4): 1047-1049 (1993); and a rat model of adult respiratory distress syndrome is described in Rabinovici *et al.*, *supra*.

Specifically contemplated by the invention are PAF-AH compositions for use in methods for treating a mammal susceptible to or suffering from PAF-mediated pathological conditions comprising administering PAF-AH to the mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF in the mammal.

Therapeutic compositions contemplated by the invention include PAF-AH and a physiologically acceptable diluent or carrier and may also include other agents having anti-inflammatory effects. Dosage amounts indicated would be sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF. For general dosage considerations see *Remmington's Pharmaceutical Sciences*, 18th Edition, Mack Publishing Co., Easton, PA (1990). Dosages will vary between about 0.1 to about 1000 µg PAF-AH/kg body weight. Therapeutic compositions of the invention may be administered by various routes depending on the pathological condition to be treated. For example, administration may be by intravenous, subcutaneous, oral, suppository, and/or pulmonary routes.

For pathological conditions of the lung, administration of PAF-AH by the pulmonary route is particularly indicated. Contemplated for use in pulmonary administration are a wide range of delivery devices including, for example, nebulizers, metered dose inhalers, and powder inhalers, which are standard in the art. Delivery of various proteins to the lungs and circulatory system by inhalation of aerosol formulations has been described in Adjei *et al.*, *Pharm. Res.*, 7(6): 565-569 (1990) (leuprolide acetate); Braquet *et al.*, *J. Cardio. Pharm.*, 13(Supp. 5): s. 143-146 (1989) (endothelin-1); Hubbard *et al.*, *Annals of Internal Medicine*, 111(3), 206-

-10-

212 (1989) (α 1-antitrypsin); Smith *et al.*, *J. Clin. Invest.*, 84: 1145-1146 (1989) (α -1-proteinase inhibitor); Debs *et al.*, *J. Immunol.*, 140: 3482-3488 (1933) (recombinant gamma interferon and tumor necrosis factor alpha); Patent Cooperation Treaty (PCT) International Publication No. WO 94/20069 published September 15, 1994
5 (recombinant pegylated granulocyte colony stimulating factor).

BRIEF DESCRIPTION OF THE DRAWING

Numerous other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof, reference being made to the drawing wherein:

10 FIGURE 1 is a photograph of a PVDF membrane containing PAF-AH purified from human plasma;

FIGURE 2 is a graph showing the enzymatic activity of recombinant human plasma PAF-AH;

15 FIGURE 3 is a schematic drawing depicting recombinant PAF-AH fragments and their catalytic activity;

FIGURE 4 is a bar graph illustrating blockage of PAF-induced rat foot edema by locally administered recombinant PAF-AH of the invention;

FIGURE 5 is a bar graph illustrating blockage of PAF-induced rat foot edema by intravenously administered PAF-AH;

20 FIGURE 6 is a bar graph showing that PAF-AH blocks PAF-induced edema but not zymosan A-induced edema;

FIGURES 7A and 7B present dose response results of PAF-AH anti-inflammatory activity in rat foot edema;

25 FIGURES 8A and 8B present results indicating the *in vivo* efficacy of a single dose of PAF-AH over time;

FIGURE 9 is a line graph representing the pharmacokinetics of PAF-AH in rat circulation; and

FIGURE 10 is a bar graph showing the anti-inflammatory effects of PAF-AH in comparison to the lesser effects of PAF antagonists in rat foot edema.

-11-

DETAILED DESCRIPTION

The following examples illustrate the invention. Example 1 presents a novel method for the purification of PAF-AH from human plasma. Example 2 describes amino acid microsequencing of the purified human plasma PAF-AH. The cloning of a full length cDNA encoding human plasma PAF-AH is described in Example 3. Identification of a putative splice variant of the human plasma PAF-AH gene is described in Example 4. The cloning of genomic sequences encoding human plasma PAF-AH is described in Example 5. Example 6 describes the cloning of canine, murine, rodent and macaque cDNAs homologous to the human plasma PAF-AH cDNA. Example 7 presents the results of an assay evidencing the enzymatic activity of recombinant PAF-AH transiently expressed in COS 7 cells. Example 8 describes the expression of human PAF-AH in *E. coli* and *S. cerevisiae*. Example 9 presents a protocol for purification of recombinant PAF-AH from *E. coli* and assays confirming its enzymatic activity. Example 10 describes various recombinant PAF-AH products including amino acid substitution analogs and amino and carboxy-truncated products. Results of a Northern blot assay for expression of human plasma PAF-AH RNA in various tissues and cell lines are presented in Example 11 while results of *in situ* hybridization are presented in Example 12. Example 13 describes the development of monoclonal antibodies specific for human plasma PAF-AH. Examples 14, 15, and 16 respectively describe the *in vivo* therapeutic effect of administration of recombinant PAF-AH products of the invention on acute inflammation, pleurisy and asthma in rats. Example 17 presents the results of immunoassays of serum of human patients exhibiting a deficiency in PAF-AH activity and describes the identification of a genetic lesion in the patients which is apparently responsible for the deficiency.

Example 1

PAF-AH was purified from human plasma in order to provide material for amino acid sequencing.

A. Optimization of Purification Conditions

Initially, low density lipoprotein (LDL) particles were precipitated from plasma with phosphotungstate and solubilized in 0.1% Tween 20 and subjected to

-12-

chromatography on a DEAE column (Pharmacia, Uppsala, Sweden) according to the method of Stafforini *et al.* (1987), *supra*, but inconsistent elution of PAF-AH activity from the DEAE column required reevaluation of the solubilization and subsequent purification conditions.

5 Tween 20, CHAPS (Pierce Chemical Co., Rockford, IL) and octyl glucoside were evaluated by centrifugation and gel filtration chromatography for their ability to solubilize LDL particles. CHAPS provided 25% greater recovery of solubilized activity than Tween 20 and 300% greater recovery than octyl glucoside. LDL precipitate solubilized with 10mM CHAPS was then fractionated on a DEAE 10 Sepharose Fast Flow column (an anion exchange column; Pharmacia) with buffer containing 1mM CHAPS to provide a large pool of partially purified PAF-AH ("the DEAE pool") for evaluation of additional columns.

15 The DEAE pool was used as starting material to test a variety of chromatography columns for utility in further purifying the PAF-AH activity. The columns tested included: Blue Sepharose Fast Flow (Pharmacia), a dye ligand affinity column; S-Sepharose Fast Flow (Pharmacia), a cation exchange column; Cu Chelating Sepharose (Pharmacia), a metal ligand affinity column; Fractogel S (EM Separations, Gibbstown, NJ), a cation exchange column; and Sephadryl-200 (Pharmacia), a gel filtration column. These chromatographic procedures all yielded 20 low, unsatisfactory levels of purification when operated in 1mM CHAPS. Subsequent gel filtration chromatography on Sephadryl S-200 in 1mM CHAPS generated an enzymatically active fraction which eluted over a broad size range rather than the expected 44 kDa approximate size. Taken together, these results indicated that the LDL proteins were aggregating in solution.

25 Different LDL samples were therefore evaluated by analytical gel filtration chromatography for aggregation of the PAF-AH activity. Samples from the DEAE pool and of freshly solubilized LDL precipitate were analyzed on Superose 12 (Pharmacia) equilibrated in buffer with 1mM CHAPS. Both samples eluted over a very broad range of molecular weights with most of the activity eluting above 150 30 kDa. When the samples were then analyzed on Superose 12 equilibrated with 10mM CHAPS, the bulk of the activity eluted near 44 kDa as expected for PAF-AH activity.

-13-

However, the samples contained some PAF-AH activity in the high molecular weight region corresponding to aggregates.

Other samples eluted PAF-AH activity exclusively in the approximately 44 kDa range when they were subsequently tested by gel filtration. These samples were an LDL precipitate solubilized in 10mM CHAPS in the presence of 0.5M NaCl and a fresh DEAE pool that was adjusted to 10mM CHAPS after elution from the DEAE column. These data indicate that at least 10mM CHAPS is required to maintain non-aggregated PAF-AH. Increase of the CHAPS concentration from 1mM to 10mM after chromatography on DEAE but prior to subsequent chromatographic steps resulted in dramatic differences in purification. For example, the degree of PAF-AH purification on S-Sepharose Fast Flow was increased from 2-fold to 10-fold. PAF-AH activity bound the Blue Sepharose Fast Flow column irreversibly in 1mM CHAPS, but the column provided the highest level of purification in 10mM CHAPS. The DEAE chromatography was not improved with prior addition of 10mM CHAPS.

Chromatography on Cu Chelating Sepharose after the Blue Sepharose Fast Flow column concentrated PAF-AH activity 15-fold. It was also determined that PAF-AH activity could be recovered from a reduced SDS-polyacrylamide gel, as long as samples were not boiled. The activity of material eluted from the Cu Chelating Sepharose column when subjected to SDS-polyacrylamide gel electrophoresis coincided with a major protein band when the gel was silver stained.

B. PAF-AH Purification Protocol

The novel protocol utilized to purify PAF-AH for amino acid sequencing therefore comprised the following steps which were performed at 4°C. Human plasma was divided into 900 ml aliquots in 1 liter Nalgene bottles and adjusted to pH 8.6. LDL particles were then precipitated by adding 90 ml of 3.85% sodium phosphotungstate followed by 23 ml of 2M MgCl₂. The plasma was then centrifuged for 15 minutes at 3600 g. Pellets were resuspended in 800 ml of 0.2% sodium citrate. LDL was precipitated again by adding 10 g NaCl and 24 ml of 2M MgCl₂. LDL particles were pelleted by centrifugation for 15 minutes at 3600 g. This wash was repeated twice. Pellets were then frozen at -20°C. LDL particles from 5L of plasma were resuspended in 5 L of buffer A (25mM Tris-HCl, 10mM CHAPS, pH 7.5) and stirred overnight. Solubilized LDL particles were centrifuged

-14-

at 3600 g for 1.5 hours. Supernatants were combined and filtered with Whatman 113 filter paper to remove any remaining solids. Solubilized LDL supernatant was loaded on a DEAE Sepharose Fast Flow column (11 cm x 10 cm; 1 L resin volume; 80 ml/minute) equilibrated in buffer B (25mM Tris-HCl, 1mM CHAPS, pH 7.5). The 5 column was washed with buffer B until absorbance returned to baseline. Protein was eluted with an 8 L, 0 - 0.5M NaCl gradient and 480 ml fractions were collected. This step was necessary to obtain binding to the Blue Sepharose Fast Flow column below. Fractions were assayed for acetylhydrolase activity essentially by the method described in Example 4.

10 Active fractions were pooled and sufficient CHAPS was added to make the pool about 10mM CHAPS. The DEAE pool was loaded overnight at 4 ml/minute onto a Blue Sepharose Fast Flow column (5 cm x 10 cm; 200 ml bed volume) equilibrated in buffer A containing 0.5M NaCl. The column was washed with the equilibration buffer at 16 ml/minute until absorbance returned to baseline. PAF-AH 15 activity was step eluted with buffer A containing 0.5M KSCN (a chaotropic salt) at 16 ml/minute and collected in 50 ml fractions. This step resulted in greater than 1000-fold purification. Active fractions were pooled, and the pool was adjusted to pH 8.0 with 1M Tris-HCl pH 8.0. The active pool from Blue Sepharose Fast Flow chromatography was loaded onto a Cu Chelating Sepharose column (2.5 cm x 2 cm; 20 10 ml bed volume; 4 ml/minute) equilibrated in buffer C [25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, pH 8.0 (pH 7.5 also worked)], and the column was washed with 50 ml buffer C. PAF-AH activity was eluted with 100 ml 50mM imidazole in buffer C and collected in 10 ml fractions. Fractions containing PAF-AH activity were pooled and dialyzed against buffer A. In addition to providing a 15-fold 25 concentration of PAF-AH activity, the Cu Chelating Sepharose column gave a small purification. The Cu Chelating Sepharose pool was reduced in 50 mM DTT for 15 minutes at 37°C and loaded onto a 0.75 mm, 7.5% polyacrylamide gel. Gel slices were cut every 0.5 cm and placed in disposable microfuge tubes containing 200 µl 25mM Tris-HCl, 10mM CHAPS, 150mM NaCl. Slices were ground up and allowed 30 to incubate overnight at 4°C. The supernatant of each gel slice was then assayed for PAF-AH activity to determine which protein band on SDS-PAGE contained PAF-AH activity. PAF-AH activity was found in an approximately 44 kDa band. Protein

-15-

from a duplicate gel was electrotransferred to a PVDF membrane (Immobilon-P, Millipore) and stained with Coomassie Blue. A photograph of the PVDF membrane is presented in FIGURE 1.

As presented in Table I below, approximately 200 µg PAF-AH was
 5 purified 2×10^6 -fold from 5 L human plasma. In comparison, a 3×10^4 -fold purification of PAF-AH activity is described in Stafforini *et al.* (1987), *supra*.

Table 1

	Sample	Vol. (mL)	Activity $\times 10^6$	Total Activity $\times 10^6$	Prot. Conc. (mg/ mL)	Specific Activity (cpm x $\times 10^6$)	% Recovery of Activity		Fold Purification	
							Step		Cum.	
							Step	Cum.	Step	Cum.
	Plasma	5000	23	116	62	0.37	100	100	1	1
10	LDL	4500	22	97	1.76	12	84	84	33	33
	DEAE	4200	49	207	1.08	46	212	178	3.7	124
	Blue	165	881	14	0.02	54200	70	126	1190	1.5×10^5
	Cu	12	12700	152	0.15	82200	104	131	1.5	2.2×10^5
	SDS-PAGE	--	--	--	--	--	--	--	-10	2.2×10^6

15 In summary, the following steps were unique and critical for successful purification of plasma PAF-AH for microsequencing: (1) solubilization and chromatography in 10mM CHAPS, (2) chromatography on a blue ligand affinity column such as Blue Sepharose Fast Flow, (3) chromatography on a Cu ligand affinity column such as Cu Chelating Sepharose, and (4) elution of PAF-AH from SDS-PAGE.

20

Example 2

For amino acid sequencing, the approximately 44 kDa protein band from the PAF-AH- containing PVDF membrane described in Example 1 was excised and sequenced using an Applied Biosystems 473A Protein sequencer. N-terminal sequence analysis of the ~44 kDa protein band corresponding to the PAF-AH

25

-16-

activity indicated that the band contained two major sequences and two minor sequences. The ratio of the two major sequences was 1:1 and it was therefore difficult to interpret the sequence data.

5 To distinguish the sequences of the two major proteins which had been resolved on the SDS gel, a duplicate PVDF membrane containing the approximately 44 kDa band was cut in half such that the upper part and the lower part of the membrane were separately subjected to sequencing.

The N-terminal sequence obtained for the lower half of the membrane was:

10 SEQ ID NO: 1

F K D L G E E N F K A L V L I A F

A search of protein databases revealed this sequence to be a fragment of human serum albumin. The upper half of the same PVDF membrane was also sequenced and the N-terminal amino acid sequence determined was:

15 SEQ ID NO: 2

I Q V L M A A A S F G Q T K I P

This sequence did not match any protein in the databases searched and was different from the N-terminal amino acid sequence:

20 SEQ ID NO: 3

M K P L V V F V L G G

which was reported for erythrocyte cytoplasmic PAF-AH in Stafforini *et al.* (1993), *supra*. The novel sequence (SEQ ID NO: 2) was utilized for cDNA cloning of human plasma PAF-AH as described below in Example 3.

Example 3

25 A full length clone encoding human plasma PAF-AH was isolated from a macrophage cDNA library.

A. Construction of a Macrophage cDNA Library

Poly A⁺ RNA was harvested from peripheral blood monocyte-derived macrophages. Double-stranded, blunt-ended cDNA was generated using the Invitrogen Copy Kit (San Diego, CA) and *Bst*XI adapters were ligated to the cDNA prior to insertion into the mammalian expression vector, pRc/CMV (Invitrogen). The

-17-

resulting plasmids were introduced into *E. coli* strain XL-1 Blue by electroporation. Transformed bacteria were plated at a density of approximately 3000 colonies per agarose plate on a total of 978 plates. Plasmid DNA prepared separately from each plate was retained in individual pools and was also combined into larger pools representing 300,000 clones each.

5 B. Library Screening by PCR

The macrophage library was screened by the polymerase chain reaction utilizing a degenerate antisense oligonucleotide PCR primer based on the novel N-terminal amino acid sequence described in Example 2. The sequence of the primer is set out below in IUPAC nomenclature and where "I" is an inosine.

SEQ ID NO: 4

5' ACATGAATTCCGGIATCYTTIGTYTGICCRAA 3'

The codon choice tables of Wada *et al.*, *Nuc. Acids Res.*, 19S: 1981-1986 (1991) were used to select nucleotides at the third position of each codon of the primer. The primer was used in combination with a primer specific for either the SP6 or T7 promoter sequences, both of which flank the cloning site of pRc/CMV, to screen the macrophage library pools of 300,000 clones. All PCR reactions contained 100 ng of template cDNA, 1 µg of each primer, 0.125mM of each dNTP, 10mM Tris-HCl pH 8.4, 50mM MgCl₂ and 2.5 units of Taq polymerase. An initial denaturation step of 20 94°C for four minutes was followed by 30 cycles of amplification of 1 minute at 94°C, 1 minute at 60°C and 2 minutes at 72°C. The resulting PCR product was cloned into pBluescript SK⁺ (Stratagene, La Jolla, CA) and its nucleotide sequence determined by the dideoxy chain termination method. The PCR product contained the sequence predicted by the novel peptide sequence and corresponds to nucleotides 25 1 to 331 of SEQ ID NO: 7.

The PCR primers set out below, which are specific for the cloned PCR fragment described above, were then designed for identifying a full length clone.

Sense Primer (SEQ ID NO: 5)

5' TATTCTAGAAGTGTGGTGGAACTCGCTGG 3'

30 Antisense Primer (SEQ ID NO: 6)

5' CGATGAATTCACTTCAGCTGCAGCAGCCATCAGTAC 3'

-18-

PCR reactions utilizing the primers were performed as described above to first screen the cDNA pools of 300,000 clones and then the appropriate subset of the smaller pools of 3000 clones. Three pools of 3000 clones which produced a PCR product of the expected size were then used to transform bacteria.

5 C. Library Screening by Hybridization

DNA from the transformed bacteria was subsequently screened by hybridization using the original cloned PCR fragment as a probe. Colonies were blotted onto nitrocellulose and prehybridized and hybridized in 50% formamide, 0.75M sodium chloride, 0.075M sodium citrate, 0.05M sodium phosphate pH 6.5, 10 1% polyvinyl pyrrolidine, 1% Ficoll, 1% bovine serum albumin and 50 ng/ml sonicated salmon sperm DNA. The hybridization probe was labeled by random hexamer priming. After overnight hybridization at 42°C, blots were washed extensively in 0.03M sodium chloride, 3mM sodium citrate, 0.1% SDS at 42°C. The nucleotide sequence of 10 hybridizing clones was determined. One of the clones, 15 clone sAH 406-3, contained the sequence predicted by the original peptide sequence of the PAF-AH activity purified from human plasma. The DNA and deduced amino acid sequences of the human plasma PAF-AH are set out in SEQ ID NOs: 7 and 8, respectively.

Clone sAH 406-3 contains a 1.52 kb insert with an open reading frame 20 that encodes a predicted protein of 441 amino acids. At the amino terminus, a relatively hydrophobic segment of 41 residues precedes the N-terminal amino acid (the isoleucine at position 42 of SEQ ID NO: 8) identified by protein microsequencing. The encoded protein may thus have either a long signal sequence or a signal sequence plus an additional peptide that is cleaved to yield the mature 25 functional enzyme. The presence of a signal sequence is one characteristic of secreted proteins. In addition, the protein encoded by clone sAH 406-3 includes the consensus GxSxG motif (amino acids 271-275 of SEQ ID NO: 8) that is believed to contain the active site serine of all known mammalian lipases, microbial lipases and serine proteases. See Chapus *et al.*, *Biochimie*, 70: 1223-1224 (1988) and Brenner, 30 *Nature*, 334: 528-530 (1988).

Table 2 below is a comparison of the amino acid composition of the human plasma PAF-AH of the invention as predicted from SEQ ID NO: 8 and the

-19-

amino acid composition of the purportedly purified material described by Stafforini *et al.* (1987), *supra*.

Table 2

		<u>Clone sAH 406-3</u>	<u>Stafforini et al.</u>
5	Ala	26	24
	Asp & Asn	48	37
	Cys	5	14
	Glu & Gln	36	42
	Phe	22	12
	Gly	29	58
	His	13	24
	Ile	31	17
	Lys	26	50
	Leu	40	26
10	Met	10	7
	Pro	15	11
	Arg	18	16
	Ser	27	36
	Thr	20	15
	Val	13	14
20	Trp	7	Not determined
	Tyr	14	13

The amino acid composition of the mature form of the human plasma PAF-AH of the invention and the amino acid composition of the previously purified material that was purportedly the human plasma PAF-AH are clearly distinct.

When alignment of the Hattori *et al.*, *supra* nucleotide and deduced amino acid sequences of bovine brain cytoplasmic PAF-AH with the nucleotide and amino acid sequences of the human plasma PAF-AH of the invention was attempted, no significant structural similarity in the sequences was observed.

-20-

Example 4

A putative splice variant of the human PAF-AH gene was detected when PCR was performed on macrophage and stimulated PBMC cDNA using primers that hybridized to the 5' untranslated region (nucleotides 31 to 52 of SEQ ID NO: 7) 5 and the region spanning the translation termination codon at the 3' end of the PAF-AH cDNA (nucleotides 1465 to 1487 of SEQ ID NO: 7). The PCR reactions yielded two bands on a gel, one corresponding to the expected size of the PAF-AH cDNA of Example 3 and the other was about 100 bp shorter. Sequencing of both bands revealed that the larger band was the PAF-AH cDNA of Example 3 while the shorter 10 band lacked exon 2 (Example 5 below) of the PAF-AH sequence which encodes the putative signal and pro-peptide sequences of plasma PAF-AH. The predicted catalytic triad and all cysteines were present in the shorter clone, therefore the biochemical activity of the protein encoded by the clone is likely to match that of the plasma enzyme.

15

Example 5

Genomic human plasma PAF-AH sequences were also isolated. The structure of the PAF-AH gene was determined by isolating lambda and P1 phage clones containing human genomic DNA by DNA hybridization under conditions of high stringency. Fragments of the phage clones were subcloned and sequenced using 20 primers designed to anneal at regular intervals throughout the cDNA clone sAH 406-3. In addition, new sequencing primers designed to anneal to the intron regions flanking the exons were used to sequence back across the exon-intron boundaries to confirm the sequences. Exon/intron boundaries were defined as the points where the genomic and cDNA sequences diverged. These analyses revealed that the human 25 PAF-AH gene is comprised of 12 exons.

30

Exons 1, 2, 3, 4, 5, 6, and part of 7 were isolated from a male fetal placental library constructed in *lambda* FIX (Stratagene). Phage plaques were blotted onto nitrocellulose and prehybridized and hybridized in 50% formamide, 0.75M sodium chloride, 75mM sodium citrate, 50mM sodium phosphate (pH 6.5), 1% polyvinyl pyrrolidine, 1% Ficoll, 1% bovine serum albumin, and 50 ng/ml sonicated salmon sperm DNA. The hybridization probe used to identify a phage clone

-21-

containing exons 2-6 and part of 7 consisted of the entire cDNA clone sAH 406-3. A clone containing exon 1 was identified using a fragment derived from the 5' end of the cDNA clone (nucleotides 1 to 312 of SEQ ID NO: 7). Both probes were labelled with ^{32}P by hexamer random priming. After overnight hybridization at 42°C, blots were washed extensively in 30mM sodium chloride, 3mM sodium citrate, 0.1% SDS at 42°C. The DNA sequences of exons 1, 2, 3, 4, 5, and 6 along with partial surrounding intron sequences are set out in SEQ ID NOS: 9, 10, 11, 12, 13, and 14, respectively.

The remainder of exon 7 as well as exons 8, 9, 10, 11, and 12 were subcloned from a P1 clone isolated from a human P1 genomic library. P1 phage plaques were blotted onto nitrocellulose and prehybridized and hybridized in 0.75M sodium chloride, 50mM sodium phosphate (pH 7.4), 5mM EDTA, 1% polyvinyl pyrrolidine, 1% Ficoll, 1% bovine serum albumin, 0.5% SDS, and 0.1 mg/ml total human DNA. The hybridization probe, labeled with ^{32}P by hexamer random priming, consisted of a 2.6 kb EcoRI fragment of genomic DNA derived from the 3' end of a lambda clone isolated above. This fragment contained exon 6 and the part of exon 7 present on the phage clone. After overnight hybridization at 65°C, blots were washed as described above. The DNA sequences of exons 7, 8, 9, 10, 11, and 12 along with partial surrounding intron sequences are set out in SEQ ID NOS: 15, 16, 17, 18, 19, and 20, respectively.

Example 6

Full length plasma PAF-AH cDNA clones were isolated from mouse and canine spleen cDNA libraries and a partial rodent clone was isolated from a rat thymus cDNA library. The clones were identified by low stringency hybridization (hybridization conditions were the same as described for exons 1 through 6 in Example 5 above except that 20% formamide instead of 50% formamide was used). A 1 kb HindIII fragment of the human PAF-AH sAH 406-3 cDNA clone (nucleotides 309 to 1322 of SEQ ID NO: 7) was used as a probe. In addition, a partial monkey clone was isolated from macaque brain cDNA by PCR using primers based on nucleotides 285 to 303 and 851 to 867 of SEQ ID NO: 7. The nucleotide and

-22-

deduced amino acid sequences of the mouse, canine, rat, and macaque cDNA clones are set out in SEQ ID NOS: 21, 22, 23, and 24, respectively.

A comparison of the deduced amino acid sequences of the cDNA clones with the human cDNA clone results in the amino acid percentage identity values set out in Table 3 below.

		<u>Human</u>	<u>Dog</u>	<u>Mouse</u>
10	Dog	80		
	Mouse	66	64	
	Monkey	92	82	69
	Rat	74	69	82

Example 7

To determine whether human plasma PAF-AH cDNA clone sAH 406-3 (Example 3) encodes a protein having PAF-AH activity, the pRc/CMV expression construct was transiently expressed in COS 7 cells. Three days following transfection by a DEAE Dextran method, COS cell media was assayed for PAF-AH activity.

Cells were seeded at a density of 300,000 cells per 60 mm tissue culture dish. The following day, the cells were incubated in DMEM containing 0.5 mg/ml DEAE dextran, 0.1mM chloroquine and 5-10 µg of plasmid DNA for 2 hours. Cells were then treated with 10% DMSO in phosphate-buffered saline for 1 minute, washed with media and incubated in DMEM containing 10% fetal calf serum previously treated with diisopropyl fluorophosphate (DFP) to inactivate endogenous bovine serum PAF-AH. After 3 days of incubation, media from transfected cells were assayed for PAF-AH activity. Assays were conducted in the presence and absence of either 10 mM EDTA or 1 mM DFP to determine whether the recombinant enzyme was calcium-independent and inhibited by the serine esterase inhibitor DFP as previously described for plasma PAF-AH by Stafforini *et al.* (1987), *supra*. Negative controls included cells transfected with pRc/CMV either lacking an insert or having the sAH 406-3 insert in reverse orientation.

PAF-AH activity in transfectant supernatants was determined by the method of Stafforini *et al.* (1990), *supra*, with the following modifications. Briefly,

-23-

PAF-AH activity was determined by measuring the hydrolysis of ^3H -acetate from [acetyl- ^3H] PAF (New England Nuclear, Boston, MA). The aqueous free ^3H -acetate was separated from labeled substrate by reversed-phase column chromatography over octadecylsilica gel cartridges (Baker Research Products, Phillipsburg, PA). Assays
5 were carried out using 10 μl transfectant supernatant in 0.1M Hepes buffer, pH 7.2, in a reaction volume of 50 μl . A total of 50 pmoles of substrate were used per reaction with a ratio of 1:5 labeled: cold PAF. Reactions were incubated for 30 minutes at 37°C and stopped by the addition of 40 μl of 10M acetic acid. The solution was then washed through the octadecylsilica gel cartridges which were then
10 rinsed with 0.1M sodium acetate. The aqueous eluate from each sample was collected and counted in a liquid scintillation counter for one minute. Enzyme activity was expressed in counts per minute.

As shown in FIGURE 2, media from cells transfected with sAH 406-3 contained PAF-AH activity at levels 4-fold greater than background. This activity
15 was unaffected by the presence of EDTA but was abolished by 1mM DFP. These observations demonstrate that clone sAH 406-3 encodes an activity consistent with the human plasma enzyme PAF-AH.

Example 8

PCR was used to generate a protein coding fragment of human plasma
20 PAF-AH cDNA from clone sAH 406-3 which was readily amenable to subcloning into an *E. coli* expression vector. The subcloned segment began at the 5' end of the human gene with the codon that encodes Ile₄₂ (SEQ ID NO: 8), the N-terminal residue of the enzyme purified from human plasma. The remainder of the gene through the native termination codon was included in the construct. The 5' sense
25 PCR primer utilized was:

SEQ ID NO: 25

5' TATTCTAGAATTATGATACAAGTATTAATGGCTGCTGCAAG

3' and contained an *Xba*I cloning site as well as a translation initiation codon (underscored). The 3' antisense primer utilized was:

30 SEQ ID NO: 26

5' ATTGATATCCTAATTGTATTCTCTATTCCCTG 3'

-24-

and encompassed the termination codon of sAH 406-3 and contained an *EcoRV* cloning site. PCR reactions were performed essentially as described in Example 3. The resulting PCR product was digested with *Xba*I and *EcoRV* and subcloned into a pBR322 vector containing the Trp promoter [deBoer *et al.*, *PNAS*, 80:21-25 (1983)] immediately upstream of the cloning site. *E. coli* strain XL-1 Blue was transformed with the expression construct and cultured in L broth containing 100 µg/ml of carbenicillin. Transformants from overnight cultures were pelleted and resuspended in lysis buffer containing 50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM CHAPS, 1mM EDTA, 100 µg/ml lysozyme, and 0.05 trypsin-inhibiting units (TIU)/ml Aprotinin. Following a 1 hour incubation on ice and sonication for 2 minutes, the lysates were assayed for PAF-AH activity by the method described in Example 4. *E. coli* transformed with the expression construct (designated trp AH) generated a product with PAF-AH activity. See Table 6 in Example 9.

Constructs including three additional promoters, the *tacII* promoter (deBoer, *supra*), the arabinose (*ara*) B promoter from *Salmonella typhimurium* [Horwitz *et al.*, *Gene*, 14: 309-319 (1981)], and the bacteriophage T7 promoter, were also utilized to drive expression of human PAF-AH sequences in *E. coli*. Constructs comprising the Trp promoter (pUC trp AH), the *tacII* promoter (pUC tac AH), and the *araB* promoter (pUC ara AH) were assembled in plasmid pUC19 (New England Biolabs, MA) while the construct comprising the T7 promoter (pET AH) was assembled in plasmid pET15B (Novagen, Madison, WI). A construct containing a hybrid promoter, pHAB/PH, consisting of the *araB* promoter fused to the ribosome binding sites of the T7 promoter region was also assembled in pET15B. All *E. coli* constructs produced PAF-AH activity within a range of 20 to 50 U/ml/OD₆₀₀. This activity corresponded to a total recombinant protein mass of ≥1% of the total cell protein.

Recombinant human PAF-AH was also been expressed in *Saccharomyces cerevisiae*. The yeast ADH2 promoter was used to drive rPAF-AH expression and produced 7 U/ml/OD₆₀₀ (Table 4 below).

-25-

Table 4

	<u>Construct</u>		<u>Strain</u>	<u>Enzyme Activity</u> <u>(U/ml/OD)</u>
5	pUC tac AH	tac	E. coli W3110	30
	pUC trp AH	trp	E. coli W3110	40
	pUC ara AH	araB	E. coli W3110	20
	pET AH	T7	E. coli BL21 (DE3) (Novagen)	50
	pHAB/PH	araB/T7	E. coli XL-1	34
	pYep ADH2 AH	ADH2	Yeast BJ2.28	7

Several *E. coli* expression constructs were also evaluated which produce PAF-AH with extended amino termini. The N-terminus of natural plasma PAF-AH was identified as Ile₄₂ by amino acid sequencing (Example 2). However, the sequence immediately upstream of Ile₄₂ does not conform to amino acids found at signal sequence cleavage sites [i.e., the "-3-1-rule" is not followed, as lysine is not found at position -1; see von Heijne, *Nuc. Acids Res.*, 14:4683-4690 (1986)]. Presumably a more classical signal sequence (M₁-A₁₇) is recognized by the cellular secretion system, followed by endoproteolytic cleavage. The entire coding sequence for PAF-AH beginning at the initiating methionine (nucleotides 162 to 1487 of SEQ ID NO: 7) was engineered for expression in *E. coli* using the trp promoter. As shown in Table 5, this construct made active PAF-AH, but expression was at about one fiftieth of the level of the original construct beginning at Ile₄₂. Another expression construct, beginning at Val₁₈ (nucleotides 213 to 1487 of SEQ ID NO: 7), produced active PAF-AH at about one third the level of the original construct. These results suggest that amino terminal end extensions are not critical or necessary for activity of recombinant PAF-AH produced in *E. coli*.

-26-

Table 5

		<u>PAF-AH activity (U/ml/OD₆₀₀)</u>	
	<u>Construct</u>	<u>Lysate</u>	<u>Media</u>
5	pUC trp AH	177.7	0.030
	pUC trp AH Met ₁	3.1	0.003
	pUC trp AH Val ₁₈	54.6	0.033

Example 9

Recombinant human plasma PAF-AH (beginning at Ile₄₂) expressed in *E. coli* was purified to a single Coomassie-stained SDS-PAGE band by various methods and assayed for activities exhibited by the native PAF-AH enzyme.

A. Purification of Recombinant PAF-AH

The first purification procedure utilized is similar to that described in Example 1 for native PAF-AH. The following steps were performed at 4°C. Pellets from 50 ml PAF-AH producing *E. coli* (transformed with expression construct trp AH) were lysed as described in Example 8. Solids were removed by centrifugation at 10,000 g for 20 minutes. The supernatant was loaded at 0.8 ml/minute onto a Blue Sepharose Fast Flow column (2.5 cm x 4 cm; 20 ml bed volume) equilibrated in buffer D (25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, pH 7.5). The column was washed with 100 ml buffer D and eluted with 100 ml buffer A containing 0.5M KSCN at 3.2 ml/minute. A 15 ml active fraction was loaded onto a 1 ml Cu Chelating Sepharose column equilibrated in buffer D. The column was washed with 5 ml buffer D followed by elution with 5 ml of buffer D containing 100mM imidazole with gravity flow. Fractions containing PAF-AH activity were analyzed by SDS-PAGE.

The results of the purification are shown in Table 6 wherein a unit equals μ mol PAF hydrolysis per hour. The purification product obtained at 4°C appeared on SDS-PAGE as a single intense band below the 43 kDa marker with some diffuse staining directly above and below it. The recombinant material is significantly more pure and exhibits greater specific activity when compared with PAF-AH preparations from plasma as described in Example 1.

-27-

Table 6

Sample	Volume (ml)	Activity (units/ ml)	Total Act. (units $\times 10^3$)	Prot Conc (mg/mL)	Specific Activity (units/ mg)	% Recovery		Fold Purification	
						Step	Cum.	Step	Cum.
Lysate	4.5	989	4451	15.6	63	100	100	1	1
Blue	15	64	960	0.07	914	22	22	14.4	14.4
5 Cu	1	2128	2128	0.55	3869	220	48	4.2	61

When the same purification protocol was performed at ambient temperature, in addition to the band below the 43 kDa marker, a group of bands below the 29 kDa marker correlated with PAF-AH activity of assayed gel slices. These lower molecular weight bands may be proteolytic fragments of PAF-AH that retain enzymatic activity.

A different purification procedure was also performed at ambient temperature. Pellets (100 g) of PAF-AH-producing *E. coli* (transformed with the expression construct pUC trp AH) were resuspended in 200 ml of lysis buffer (25mM Tris, 20mM CHAPS, 50mM NaCl, 1mM EDTA, 50 µg/ml benzamidine, pH 7.5) and lysed by passing three times through a microfluidizer at 15,000 psi. Solids were removed by centrifugation at 14,300 x g for 1 hour. The supernatant was diluted 10-fold in dilution buffer [25mM MES (2-[N-morpholino] ethanesulfonic acid), 10mM CHAPS, 1mM EDTA, pH 4.9] and loaded at 25 ml/minute onto an S Sepharose Fast Flow Column (200 ml) (a cation exchange column) equilibrated in Buffer E (25mM MES, 10mM CHAPS, 1mM EDTA, 50mM NaCl, pH 5.5). The column was washed with 1 liter of Buffer E, eluted with 1M NaCl, and the eluate was collected in 50 ml fractions adjusted to pH 7.5 with 0.5 ml of 2M Tris base. Fractions containing PAF-AH activity were pooled and adjusted to 0.5M NaCl. The S pool was loaded at 1 ml/minute onto a Blue Sepharose Fast Flow column (2.5 cm x 4 cm; 20 ml) equilibrated in Buffer F (25mM Tris, 10mM CHAPS, 0.5M NaCl, 1mM EDTA, pH 7.5). The column was washed with 100 ml Buffer F and eluted with 100 ml Buffer F containing 3M NaCl at 4 ml/minute. The Blue Sepharose Fast Flow chromatography step was then repeated to reduce endotoxin levels in the sample.

-28-

Fractions containing PAF-AH activity were pooled and dialyzed against Buffer G (25mM Tris pH 7.5, 0.5M NaCl, 0.1% Tween 80, 1mM EDTA).

The results of the purification are shown in Table 7 wherein a unit equals μ mol PAF hydrolysis per hour.

5

Table 7

Sample	Volume (ml)	Activity (units/ ml)	Total Act. (units $\times 10^3$)	Prot Conc (mg/ml)	Specific Activity (units/ mg)	% Recovery of Activity Step	% Recovery of Activity Cum.	Fold Purification Step	Fold Purification Cum.
Lysate	200	5640	1128	57.46	98	100	100	1	1
S	111	5742	637	3.69	1557	57	56	16	16
Blue	100	3944	394	0.84	4676	35	62	3	48

10 The purification product obtained appeared on SDS-PAGE as a single intense band below the 43 kDa marker with some diffuse staining directly above and below it. The recombinant material is significantly more pure and exhibits greater specific activity when compared with PAF-AH preparations from plasma as described in Example 1.

15 Yet another purification procedure contemplated by the present invention involves the following cell lysis, clarification, and first column steps. Cells are diluted 1:1 in lysis buffer (25mM Tris pH 7.5, 150mM NaCl, 1% Tween 80, 2mM EDTA). Lysis is performed in a chilled microfluidizer at 15,000-20,000 psi with three passes of the material to yield >99% cell breakage. The lysate is diluted 20 1:20 in dilution buffer (25mM Tris pH 8.5, 1mM EDTA) and applied to a column packed with Q-Sepharose Big Bead chromatography media (Pharmacia) and equilibrated in 25mM Tris pH 8.5, 1mM EDTA, 0.015% Tween 80. The eluate is diluted 1:10 in 25mM MES pH 5.5, 1.2M Ammonium sulfate, 1mM EDTA and applied to Butyl Sepharose chromatography media (Pharmacia) equilibrated in the same buffer. PAF-AH activity is eluted in 25mM MES pH. 5.5, 0.1% Tween 80, 1mM EDTA.

B. Activity of Recombinant PAF-AH

The most remarkable property of the PAF acetylhydrolase is its marked specificity for substrates with a short residue at the *sn*-2 position of the substrate. This strict specificity distinguishes PAF acetylhydrolase from other forms of PLA₂.
5 Thus, to determine if recombinant PAF-AH degrades phospholipids with long-chain fatty acids at the *sn*-2 position, hydrolysis of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (arachidonoylPC) was assayed since this is the preferred substrate for a well-characterized form of PLA₂. As predicted from previous studies with native PAF-AH, this phospholipid was not hydrolyzed when incubated with recombinant PAF-AH. In additional experiments, arachidonoylPC was included in a standard PAF hydrolysis assay at concentrations ranging from 0 to 125 μ M to determine whether it inhibited the hydrolysis of PAF by recombinant PAF-AH. There was no inhibition of PAF hydrolysis even at the highest concentration of PAF-AH, which was 5-fold greater than the concentration of PAF. Thus, recombinant PAF-AH exhibits the same
10 substrate selectivity as the native enzyme; long chain substrates are not recognized. Moreover, recombinant PAF-AH enzyme rapidly degraded an oxidized phospholipid (glutaroylPC) which had undergone oxidative cleavage of the *sn*-2 fatty acid. Native plasma PAF-AH has several other properties that distinguish it from other phospholipases including calcium-independence and resistance to compounds that modify sulphhydryl groups or disrupt disulfides.
15
20

Both the native and recombinant plasma PAF-AH enzymes are sensitive to DFP, indicating that a serine comprises part of their active sites. An unusual feature of the native plasma PAF acetylhydrolase is that it is tightly associated with lipoproteins in circulation, and its catalytic efficiency is influenced by the lipoprotein environment. When recombinant PAF-AH of the invention was incubated with human plasma (previously treated with DFP to abolish the endogenous enzyme activity), it associated with low and high density lipoproteins in the same manner as the native activity. This result is significant because there is substantial evidence that modification of low density lipoproteins is essential for the cholesterol deposition observed in atherosomas, and that oxidation of lipids is an initiating factor in this process. PAF-AH protects low density lipoproteins from modification under oxidizing conditions *in vitro* and may have such a role *in vivo*. Administration of
25
30

-30-

PAF-AH is thus indicated for the suppression the oxidation of lipoproteins in atherosclerotic plaques as well as to resolve inflammation.

These results all confirm that the cDNA clone sAH 406-3 encodes a protein with the activities of the the human plasma PAF acetylhydrolase.

5

Example 10

Various other recombinant PAF-AH products were expressed in *E. coli*. The products included PAF-AH analogs having single amino acid mutations and PAF-AH fragments.

10 **A. PAF-AH Amino Acid Substitution Products**

PAF-AH is a lipase because it hydrolyses the phospholipid PAF. While no obvious overall similarity exists between PAF-AH and other characterized lipases, there are conserved residues found in comparisons of structurally characterized lipases. A serine has been identified as a member of the active site. The serine, along with an aspartate residue and a histidine residue, form a catalytic triad which represents the active site of the lipase. The three residues are not adjacent in the primary protein sequence, but structural studies have demonstrated that the three residues are adjacent in three dimensional space. Comparisons of structures of mammalian lipases suggest that the Asp residue is generally twenty-four amino acids C-terminal to the active site serine. In addition, the histidine is generally 109 to 111 amino acids C-terminal to the active site serine.

By site-directed mutagenesis and PCR, individual codons of the human PAF-AH coding sequence were modified to encode alanine residues and were expressed in *E. coli*. As shown in Table 8 below wherein, for example, the abbreviation "S108A" indicates that the serine residue at position 273 was changed to an alanine, point mutations of Ser₂₇₃, Asp₂₉₆, or His₃₅₁ completely destroy PAF-AH activity. The distances between active site residues is similar for PAF-AH (Ser to Asp, 23 amino acids; Ser to His, 78 amino acids) and other lipases. These experiments demonstrate that Ser₂₇₃, Asp₂₉₆, and His₃₅₁ are critical residues for activity and are therefore likely candidates for catalytic triad residues. Cysteines are often critical for the functional integrity of proteins because of their capacity to form disulfide bonds. The plasma PAF-AH enzyme contains five cysteines. To determine

-31-

whether any of the five is critical for enzyme activity, each cysteine was mutated individually to a serine and the resulting mutants were expressed in *E. coli*. As shown below in Table 8, a significant but not total loss of PAF-AH activity resulted from the conversion of either Cys₂₂₉ or Cys₂₉₁ to serine. Therefore, these cysteines appear to be necessary for full PAF-AH activity. Other point mutations had little or no effect on PAF-AH catalytic activity. In Table 8, "++++" represent wild type PAF-AH activity of about 40-60 U/ml/OD₆₀₀, "+++" represents about 20-40 U/ml/OD₆₀₀ activity, "+" represents about 10-20 U/ml/OD₆₀₀ activity, "+" represents 1-10 U/ml/OD₆₀₀ activity, and "-" indicates <1 U/ml/OD₆₀₀ activity.

10

Table 8

	<u>Mutation</u>	<u>PAF-AH activity</u>
	Wild type	++++
	S108A	++++
	S273A	-
15	D296A	-
	D338A	++++
	H351A	-
	H395A, H399A	++++
	C67S	+++
20	C229S	+
	C291S	+
	C334S	++++
	C407S	+++

-32-

B. PAF-AH Fragment Products

C-terminal deletions were prepared by digesting the 3' end of the PAF-AH coding sequence with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. Ten different deletion constructs were characterized by DNA sequence analysis, protein expression, and PAF-AH activity. Removal of twenty-one to thirty C-terminal amino acids greatly reduced catalytic activity and removal of fifty-two residues completely destroyed activity. See FIGURE 3.

Similar deletions were made at the amino terminal end of PAF-AH. Fusions of PAF-AH with *E. coli* thioredoxin at the N-terminus were prepared to facilitate consistent high level expression PAF-AH activity [LaVallie *et al.*, *Bio/technology*, 11:187-193 (1993)]. Removal of nineteen amino acids from the naturally processed N-terminus (Ile₄₂) completely destroyed enzymatic activity in the fusion protein. See FIGURE 3.

15

Example 11

A preliminary analysis of expression patterns of human plasma PAF-AH mRNA in human tissues was conducted by Northern blot hybridization.

RNA was prepared from human cerebral cortex, heart, kidney, placenta, thymus and tonsil using RNA Stat 60 (Tel-Test "B", Friendswood, TX). Additionally, RNA was prepared from the human hematopoietic precursor-like cell line, THP-1 (ATCC TIB 202), which was induced to differentiate to a macrophage-like phenotype using the phorbol ester phorbolmyristylacetate (PMA). Tissue RNA and RNA prepared from the premyelocytic THP-1 cell line prior to and 1 to 3 days after induction were electrophoresed through a 1.2% agarose formaldehyde gel and subsequently transferred to a nitrocellulose membrane. The full length human plasma PAF-AH cDNA, sAH 406-3, was labelled by random priming and hybridized to the membrane under conditions identical to those described in Example 3 for library screening. Initial results indicate that the PAF-AH probe hybridized to a 1.8 kb band in the thymus, tonsil, and to a lesser extent, the placental RNA.

30

The expression of PAF-AH RNA in monocytes isolated from human blood and during their spontaneous differentiation into macrophages in culture was

-33-

also examined. Little or no RNA was detected in fresh monocytes, but expression was induced and maintained during differentiation into macrophages. There was a concomitant accumulation of PAF-AH activity in the culture medium of the differentiating cells. Expression of the human plasma PAF-AH transcript was also observed in the THP-1 cell RNA at 1 day but not 3 days following induction. THP-1 cells did not express mRNA for PAF-AH in the basal state.

Example 12

PAF-AH expression in human and mouse tissues was examined by *in situ* hybridization.

Human tissues were obtained from National Disease Research Interchange and the Cooperative Human Tissue Network. Normal mouse brain and spinal cord, and EAE stage 3 mouse spinal cords were harvested from S/JLJ mice. Normal S/JLJ mouse embryos were harvested from eleven to eighteen days after fertilization.

The tissue sections were placed in Tissue Tek II cryomolds (Miles Laboratories, Inc., Naperville, IL) with a small amount of OCT compound (Miles, Inc., Elkhart, IN). They were centered in the cryomold, the cryomold filled with OCT compound, then placed in a container with 2-methylbutane [$C_2H_5CH(CH_3)_2$, Aldrich Chemical Company, Inc., Milwaukee, WI] and the container placed in liquid nitrogen. Once the tissue and OCT compound in the cryomold were frozen, the blocks were stored at -80°C until sectioning. The tissue blocks were sectioned at 6 μ m thickness and adhered to Vectabond (Vector Laboratories, Inc., Burlingame, CA) coated slides and stored at -70°C and placed at 50°C for approximately 5 minutes to warm them and remove condensation and were then fixed in 4% paraformaldehyde for 20 minutes at 4°C, dehydrated (70%, 95%, 100% ethanol) for 1 minute at 4°C in each grade, then allowed to air dry for 30 minutes at room temperature. Sections were denatured for 2 minutes at 70°C in 70% formamide/2X SSC, rinsed twice in 2X SSC, dehydrated and then air dried for 30 minutes. The tissues were hybridized *in situ* with radiolabeled single-stranded mRNA generated from DNA derived from an internal 1 Kb HindIII fragment of the PAF-AH gene (nucleotides 308 to 1323 of SEQ ID NO: 7) by *in vitro* RNA transcription incorporation ^{35}S -UTP (Amersham).

-34-

The probes were used at varying lengths from 250-500 bp. Hybridization was carried out overnight (12-16 hours) at 50°C; the 35 S-labeled riboprobes (6×10^5 cpm/section), tRNA (0.5 μ g/section) and diethylpyrocarbonate (depc)-treated water were added to hybridization buffer to bring it a final concentration of 50% formamide, 0.3M NaCl, 5 20 mM Tris pH 7.5, 10% dextran sulfate, 1X Denhardt's solution, 100 mM dithiothreitol (DTT) and 5 mM EDTA. After hybridization, sections were washed for 1 hour at room temperature in 4X SSC/10 mM DTT, then for 40 minutes at 60°C in 10 50% formamide/1X SSC/10 mM DTT, 30 minutes at room temperature in 2X SSC, and 30 minutes at room temperature in 0.1X SSC. The sections were dehydrated, air dried for 2 hours, coated with Kodak NTB2 photographic emulsion, air dried for 2 hours, developed (after storage at 4°C in complete darkness) and counterstained with hematoxylin/eosin.

A. Brain

15 *Cerebellum.* In both the mouse and the human brains, strong signal was seen in the Purkinje cell layer of the cerebellum, as well as on individual neuronal cell bodies in the dentate nucleus (one of the four deep nuclei in the cerebellum). Additionally, signal was seen on individual cells in the granular and molecular layers of the grey matter.

20 *Hippocampus.* In the human hippocampus section, individual cells throughout the section, which appear to be neuronal cell bodies, showed strong signal.

Brain stem. On both human and mouse brain stem sections, there was strong signal on individual cells in the grey matter.

25 *Cortex.* On human cortex sections taken from the cerebral, occipital, and temporal cortices, and on mouse whole brain sections, individual cells throughout the cortex showed strong signal. There does not appear to be differentiation in the expression pattern in the different layers of the cortex. These *in situ* hybridization results are different from the results for cerebral cortex obtained by Northern blotting. The difference is likely to result from the greater sensitivity of *in situ* hybridization compared to that of Northern blotting.

30 *Pituitary.* Somewhat weak signal was seen on scattered individual cells in the pars distalis of the human tissue section.

-35-

B. Human colon

Both normal and Crohn's disease colons displayed signal in the lymphatic aggregations present in the mucosa of the sections, with the level of signal being slightly higher in the section from the Crohn's disease patient. The Crohn's disease colon also had strong signal in the lamina propria. Similarly, a high level of signal was observed in a diseased appendix section while the normal appendix exhibited a lower but still detectable signal. The sections from the ulcerative colitis patient showed no evident signal in either the lymphatic aggregations or the lamina propria.

10 C. Human tonsil and thymus

Strong signal was seen on scattered groups of individual cells within the germinal centers of the tonsil and within the thymus.

D. Human lymph node

Strong signal was observed on the lymph node section taken from a normal donor, while somewhat weak signal was observed in the lymph nodules of the section from a donor with septic shock.

E. Human small intestine

Both normal and Crohn's disease small intestine had weak signal in the Peyer's patches and lamina propria in the sections, with the signal on the diseased tissue slightly higher.

F. Human spleen and lung

Signal was not observed on any of the spleen (normal and splenic abcess sections) or lung (normal and emphysema sections) tissues.

G. Mouse spinal cord

In both the normal and EAE stage 3 spinal cords, there was strong signal in the grey matter of the spinal cord, with the expression being slightly higher in the EAE stage 3 spinal cord. In the EAE stage 3 spinal cord, cells in the white matter and perivascular cuffs, probably infiltrating macrophages and/or other leukocytes, showed signal which was absent in the normal spinal cord.

30 F. Mouse embryos

In the day 11 embryo signal was apparent in the central nervous system in the fourth ventricle, which remained constant throughout the embryo time course

-36-

as it developed into the cerebellum and brain stem. As the embryos matured, signal became apparent in central nervous system in the spinal cord (day 12), primary cortex and ganglion Gasseri (day 14), and hypophysis (day 16). Signal was observed in the peripheral nervous system (beginning on day 14 or 15) on nerves leaving the spinal cord, and, on day 17, strong signal appeared around the whiskers of the embryo. Expression was also seen in the liver and lung at day 14, the gut (beginning on day 15), and in the posterior portion of the mouth/throat (beginning on day 16). By day 18, the expression pattern had differentiated into signal in the cortex, hindbrain (cerebellum and brain stem), nerves leaving the lumbar region of the spinal cord, the posterior portion of the mouth/throat, the liver, the kidney, and possible weak signal in the lung and gut.

10 G. Summary

PAF-AH mRNA expression in the tonsil, thymus, lymph node, Peyer's patches, appendix, and colon lymphatic aggregates is consistent with the conclusions
15 that the probable predominant *in vivo* source of PAF-AH is the macrophage because these tissues all are populated with tissue macrophages that serve as phagocytic and antigen-processing cells.

20 Expression of PAF-AH in inflamed tissues would be consistent with the hypothesis that a role of monocyte-derived macrophages is to resolve inflammation. PAF-AH would be expected to inactivate PAF and the pro-inflammatory phospholipids, thus down-regulating the inflammatory cascade of events initiated by these mediators.

25 PAF has been detected in whole brain tissue and is secreted by rat cerebellar granule cells in culture. *In vitro* and *in vivo* experiments have demonstrated that PAF binds a specific receptor in neural tissues and induces functional and phenotypic changes such as calcium mobilization, upregulation of transcription activating genes, and differentiation of the neural precursor cell line, PC12. These observations suggested a physiologic role for PAF in the brain, and consistent with this, recent experiments using hippocampal tissue section cultures and
30 PAF analogs and antagonists have implicated PAF as an important retrograde messenger in hippocampal long term potentiation. Therefore, in addition to its pathological effect in inflammation, PAF appears to participate in routine neuronal

-37-

signalling processes. Expression of the extracellular PAF-AH in the brain may serve to regulate the duration and magnitude of PAF-mediated signalling.

Example 13

5 Monoclonal antibodies specific for recombinant human plasma PAF-AH were generated using *E. coli* produced PAF-AH as an immunogen.

10 Mouse #1342 was injected on day 0, day 19, and day 40 with recombinant PAF-AH. For the prefusion boost, the mouse was injected with the immunogen in PBS, four days later the mouse was sacrificed and its spleen removed steriley and placed in 10ml serum free RPMI 1640. A single-cell suspension was formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension was filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum free RPMI. Thymocytes taken from 3 naive Balb/c mice were prepared in a similar manner. NS-1 myeloma cells, kept in log phase in RPMI with 11% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, were centrifuged at 200 g for 5 minutes, and the pellet was washed twice as described in the foregoing paragraph.

15 One $\times 10^8$ spleen cells were combined with 2.0×10^7 NS-1 cells, centrifuged and the supernatant was aspirated. The cell pellet was dislodged by tapping the tube and 1 ml of 37°C PEG 1500 (50% in 75mM Hepes, pH 8.0) (Boehringer Mannheim) was added with stirring over the course of 1 minute, followed by adding 7 ml of serum free RPMI over 7 minutes. An additional 8 ml RPMI was added and the cells were centrifuged at 200 g for 10 minutes. After discarding the supernatant, the pellet was resuspended in 200 ml RPMI containing 15% FBS, 100 µM sodium hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5×10^6 thymocytes/ml and plated into 10 Corning flat bottom 96 well tissue culture plates (Corning, Corning New York).

-38-

On days 2, 4, and 6, after the fusion, 100 μ l of medium was removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion was screened by ELISA, testing for the presence of mouse IgG binding to recombinant PAF-AH. Immulon 4 plates (Dynatech, Cambridge, MA) were coated 5 for 2 hours at 37°C with 100 ng/well recombinant PAF-AH diluted in 25mM TRIS, pH 7.5. The coating solution was aspirated and 200ul/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] was added and incubated for 30 minutes at 37°C. Plates were washed three times with PBS with 0.05% Tween 20 (PBST) and 50 μ l culture supernatant was added. After incubation at 37°C for 30 10 minutes, and washing as above, 50 μ l of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST was added. Plates were incubated as above, washed four times with PBST and 100 μ L substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 μ l/ml 30% H₂O₂ in 100 mM Citrate, pH 4.5, was added. The color reaction was 15 stopped in 5 minutes with the addition of 50 μ l of 15% H₂SO₄. A₄₉₀ was read on a plate reader (Dynatech).

Selected fusion wells were cloned twice by dilution into 96 well plates and visually scoring the number of colonies/well after 5 days. Hybridomas cloned 20 were 90D1E, 90E3A, 90E6C, 90G11D (ATCC HB 11724), and 90F2D (ATCC HB 11725).

The monoclonal antibodies produced by hybridomas were isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, IN). Results showed that the monoclonal antibodies produced by hybridomas from fusion 90 were all IgG₁.

Example 14

Experimental studies were performed to evaluate the *in vivo* therapeutic 25 effects of recombinant PAF-AH of the invention on acute inflammation using a rat foot edema model [Henriques *et al.*, *Br. J. Pharmacol.*, 106: 579-582 (1992)]. The results of these studies demonstrated that PAF-AH blocks PAF-induced edema. Parallel studies were done to compare the effectiveness of PAF-AH with two 30 commercially available PAF antagonists.

-39-

A. Preparation of PAF-AH

5 *E. coli* transformed with the PAF-AH expression vector puc trp AH were lysed in a microfluidizer, solids were centrifuged out and the cell supernatants were loaded onto a S-Sepharose column (Pharmacia). The column was washed
10 extensively with buffer consisting of 50mM NaCl, 10mM CHAPS, 25mM MES and 1mM EDTA, pH 5.5. PAF-AH was eluted by increasing the NaCl concentration of the buffer to 1M. Affinity chromatography using a Blue Sepharose column (Pharmacia) was then used as an additional purification step. Prior to loading the PAF-AH preparation on the Blue Sepharose column, the sample was diluted 1:2 to reduce the NaCl concentration to 0.5M and the pH was adjusted to 7.5. After washing the Blue Sepharose column extensively with buffer consisting of 0.5M NaCl, 25mM tris, 10mM CHAPS and 1mM EDTA, pH 7.5 the PAF-AH was eluted by increasing the NaCl concentration to 3.0M.

15 Purity of PAF-AH isolated in this manner was generally 95% as assessed by SDS-PAGE with activity in the range of 5000-10,000 U/ml. Additional quality controls done on each PAF-AH preparation included determining endotoxin levels and hemolysis activity on freshly obtained rat erythrocytes. A buffer containing 25mM Tris, 10mM CHAPS, 0.5M NaCl, pH 7.5 functioned as storage media of the enzyme as well as carrier for administration. Dosages used in
20 experiments were based on enzyme activity assays conducted immediately prior to experiments.

B. Induction of Edema

25 Six to eight-week-old female Long Evans rats (Charles River, Wilmington, MA), weighing 180-200 grams, were used for all experiments. Prior to experimental manipulations, animals were anesthetized with a mixture of the anesthetics Ketaset (Fort Dodge Laboratories, Fort Dodge, IA), Rompun (Miles, Shawnee Mission, KS), and Ace Promazine (Aveco, Fort Dodge, IA) administered subcutaneously at approximately 2.5 mg Ketaset, 1.6 mg Rompun, 0.2 mg Ace Promazine per animal per dose. Edema was induced in the foot by administration of either PAF or zymosan as follows. PAF (Sigma #P-1402) was freshly prepared for each experiment from a 19.1mM stock solution stored in chloroform/methanol (9:1) at -20°C. Required volumes were dried down under N₂, diluted 1:1000 in a buffer
30

-40-

containing 150mM NaCl, 10mM Tris pH 7.5, and 0.25% BSA, and sonicated for five minutes. Animals received 50 μ l PAF (final dose of 0.96 nmoles) subcutaneously between the hind foot pads, and edema was assessed after 1 hour and again after 2 hours in some experiments. Zymosan A (Sigma #A-8800) was freshly prepared for each experiment as a suspension of 10 mg/ml in PBS. Animals received 50 μ l of zymosan (final dose of 500 μ g) subcutaneously between the hind foot pads and edema was assessed after 2 hours.

Edema was quantitated by measuring the foot volume immediately prior to administration of PAF or zymosan and at indicated time point post-challenge with PAF or zymosan. Edema is expressed as the increase in foot volume in milliliters. Volume displacement measurements were made on anesthetized animals using a plethysmometer (UGO Basile, model #7150) which measures the displaced water volume of the immersed foot. In order to insure that foot immersion was comparable from one time point to the next, the hind feet were marked in indelible ink where the hairline meets the heel. Repeated measurements of the same foot using this technique indicate the precision to be within 5%.

15 C. PAF-AH Administration Routes and Dosages

PAF-AH was injected locally between the foot pads, or systematically by IV injection in the tail vein. For local administration rats received 100 μ l PAF-AH (4000-6000 U/ml) delivered subcutaneously between the right hind foot pads. Left feet served as controls by administration of 100 μ l carrier (buffered salt solution). For systemic administration of PAF-AH, rats received the indicated units of PAF-AH in 300 μ l of carrier administered IV in the tail vein. Controls received the appropriate volume of carrier IV in the tail vein.

25 D. Local Administration of PAF-AH

Rats (N=4) were injected with 100 μ l of PAF-AH (4000-6000 U/ml) subcutaneously between the right foot pads. Left feet were injected with 100 μ l carrier (buffered salt solution). Four other rats were injected only with carrier. All rats were immediately challenged with PAF via subcutaneous foot injection and foot volumes assessed 1 hour post-challenge. FIGURE 4, wherein edema is expressed as average increase in foot volume (ml) \pm SEM for each treatment group, illustrates that PAF-induced foot edema is blocked by local administration of PAF-AH. The group

-41-

which received local PAF-AH treatment prior to PAF challenge showed reduced inflammation compared to the control injected group. An increase in foot volume of 0.08 ml \pm 0.08 (SEM) was seen in the PAF-AH group as compared to 0.63 \pm 0.14 (SEM) for the carrier treated controls. The increase in foot volume was a direct result of PAF injection as animals injected in the foot only with carrier did not exhibit an increase in foot volume.

5 E. Intravenous Administration of PAF-AH

Rats (N=4 per group) were pretreated IV with either PAF-AH (2000 U in 300 μ l carrier) or carrier alone, 15 minutes prior to PAF challenge. Edema was assessed 1 and 2 hours after PAF challenge. FIGURE 5, wherein edema is expressed as average increase in volume (ml) \pm SEM for each treatment group, illustrates that IV administration of PAF-AH blocked PAF induced foot edema at one and two hours post challenge. The group which received 2000 U of PAF-AH given by the IV route showed a reduction in inflammation over the two hour time course. Mean volume increase for the PAF-AH treated group at two hours was 0.10 ml \pm 0.08 (SEM), versus 0.56 ml \pm 0.11 for carrier treated controls.

10 F. Comparison of PAF-AH Protection in Edema Induced by PAF or Zymosan

Rats (N=4 per group) were pretreated IV with either PAF-AH (2000 U in 300 μ l carrier) or carrier alone. Fifteen minutes after pretreatment, groups received either PAF or zymosan A, and foot volume was assessed after 1 and 2 hours, respectively. As shown in FIGURE 6, wherein edema is expressed as average increase in volume (ml) \pm SEM for each treatment group, systemic administration of PAF-AH (2000 U) was effective in reducing PAF-induced foot edema, but failed to block zymosan induced edema. A mean increase in volume of 0.08 \pm 0.02 was seen in the PAF-AH treated group versus 0.49 \pm 0.03 for the control group.

15 G. Effective Dose Titration of PAF-AH Protection

In two separate experiments, groups of rats (N=3 to 4 per group) were pretreated IV with either serial dilutions of PAF-AH or carrier control in a 300 μ l volume, 15 minutes prior to PAF challenge. Both feet were challenged with PAF (as described above) and edema was assessed after 1 hour. FIGURE 7 wherein edema is expressed as average increase in volume (ml) \pm SEM for each treatment group, illustrates the increase in protection from PAF-induced edema in rats injected with

-42-

increasing dosages of PAF-AH. In the experiments, the ID₅₀ of PAF-AH given by the IV route was found to be between 40 and 80 U per rat.

H. In Vivo Efficacy of PAF-AH as a Function of Time After Administration

5 In two separate experiments, two groups of rats (N=3 to 4 per group) were pretreated IV with either PAF-AH (2000 U in 300 μ l carrier) or carrier alone. After administration, groups received PAF at time points ranging from 15 minutes to 47 hours post PAF-AH administration. Edema was then assessed 1 hour after PAF challenge. As shown in FIGURE 8, wherein edema is expressed as average increase in volume (ml) \pm SEM for each treatment group, administration of 2000 U of PAF-AH protects rats from PAF induced edema for at least 24 hours.

10

I. Pharmacokinetics of PAF-AH

15 Four rats received 2000 U of PAF-AH by IV injection in a 300 μ l volume. Plasma was collected at various time points and stored at 4°C and plasma concentrations of PAF-AH were determined by ELISA using a double mAb capture assay. In brief, monoclonal antibody 90G11D (Example 13) was diluted in 50mM carbonate buffer pH 9.6 at 100 ng/ml and immobilized on Immulon 4 ELISA plates overnight at 4°C. After extensive washing with PBS containing 0.05% Tween 20, the plates were blocked for 1 hour at room temperature with 0.5% fish skin gelatin (Sigma) diluted in PBS. Serum samples diluted in PBS with 15mM CHAPS were
20 added in duplicate to the washed ELISA plate and incubated for 1 hour at room temperature. After washing, a biotin conjugate of monoclonal antibody 90F2D (Example 13) was added to the wells at a concentration of 5 μ g/ml diluted in PBS and incubated for 1 hour at room temperature. After washing, 50 μ l of a 1:1000 dilution of ExtraAvidin (Sigma) was added to the wells and incubated for 1 hour at room
25 temperature. After washing, wells were developed using OPD as a substrate and quantitated. Enzyme activity was then calculated from a standard curve. FIGURE 9, wherein data points represent means \pm SEM, shows that at one hour plasma enzyme levels approached the predicted concentration based on a 5-6 ml plasma volume for 180-200 gram rats, mean = 374 U/ml \pm 58.2. Beyond one hour plasma
30 levels steadily declined, reaching a mean plasma concentration of 19.3 U/ml \pm 3.4 at 24 hours, which is still considerably higher than endogenous rat PAF-AH levels which have been found to be approximately 4 U/ml by enzymatic assays.

-43-

J. Effectiveness of PAF-AH Versus PAF Antagonists

Groups of rats (N=4 per group) were pretreated with one of three potential antiinflammatories: the PAF antagonist CV3988 (Biomol #L-103) administered IP (2 mg in 200 μ l EtOH), the PAF antagonist Alprazolam (Sigma #A-8800) administered IP (2 mg in 200 μ l EtOH), or PAF-AH (2000 U) administered IV. Control rats were injected IV with a 300 μ l volume of carrier. The PAF antagonists were administered IP because they are solubilized in ethanol. Rats injected with either CV3988 or Alprazolam were challenged with PAF 30 minutes after administration of the PAF antagonist to allow the PAF antagonist to enter circulation, while PAF-AH and carrier-treated rats were challenged 15 minutes after enzyme administration. Rats injected with PAF-AH exhibited a reduction in PAF-induced edema beyond that afforded by the established PAF antagonists CV3988 and Alprazolam. See FIGURE 10 wherein edema is expressed as average increase in volume (ml) \pm SEM for each treatment group.

In summary, PAF-AH is effective in blocking edema mediated by PAF *in vivo*. Administration of PAF-AH can be either local or systemic by IV injection. In dosing studies, IV injections in the range of 160-2000 U/rat were found to dramatically reduce PAF mediated inflammation, while the ID₅₀ dosage appears to be in the range of 40-80 U/rat. Calculations based on the plasma volume for 180-200 gram rats predicts that a plasma concentration in the range of 25-40 U/ml should block PAF-elicited edema. These predictions are supported by preliminary pharmacokinetic studies. A dosage of 2000 U of PAF-AH was found to be effective in blocking PAF mediated edema for at least 24 hours. At 24 hours following administration of PAF-AH plasma concentrations of the enzyme were found to be approximately 25 U/ml. PAF-AH was found to block PAF-induced edema more effectively than the two known PAF antagonists tested.

Collectively, these results demonstrate that PAF-AH effectively blocks PAF induced inflammation and may be of therapeutic value in diseases where PAF is the primary mediator.

-44-

Example 15

Recombinant PAF-AH of the invention was tested in a second *in vivo* model, PAF-induced pleurisy. PAF has previously been shown to induce vascular leakage when introduced into the pleural space [Henriques *et al.*, *supra*]. Female rats (Charles River, 180-200 g) were injected in the tail vein with 200 μ l of 1% Evans blue dye in 0.9% with 300 μ l recombinant PAF-AH (1500 μ mol/ml/hour, prepared as described in Example 14) or with an equivalent volume of control buffer. Fifteen minutes later the rats received an 100 μ l injection of PAF (2.0 nmol) into the pleural space. One hour following PAF challenge, rats were sacrificed and the pleural fluid was collected by rinsing the cavity with 3 ml heparinized phosphate buffered saline. The degree of vascular leak was determined by the quantity of Evans blue dye in the pleural space which was quantitated by absorbance at 620 nm. Rats pretreated with PAF-AH were found to have much less vascular leakage than control animals (representing more than an 80% reduction in inflammation).

The foregoing results support the treatment of subjects suffering from pleurisy with recombinant PAF-AH enzyme of the invention.

Example 16

Recombinant PAF-AH enzyme of the invention was also tested for efficacy in a model of antigen-induced eosinophil recruitment. The accumulation of eosinophils in the airway is a characteristic feature of late phase immune responses which occur in asthma, rhinitis and eczema. BALB/c mice (Charles River) were sensitized by two intraperitoneal injections consisting of 1 μ g of ovalbumin (OVA) in 4 mg of aluminum hydroxide (Inject alum, Pierce Laboratories, Rockford, IL) given at a 2 week interval. Fourteen days following the second immunization, the sensitized mice were challenged with either aerosolized OVA or saline as a control.

Prior to challenge mice were randomly placed into four groups, with four mice/group. Mice in groups 1 and 3 were pretreated with 140 μ l of control buffer consisting of 25mM tris, 0.5M NaCl, 1mM EDTA and 0.1% Tween 80 given by intravenous injection. Mice in groups 2 and 4 were pretreated with 750 units of PAF-AH (activity of 5,500 units/ml given in 140 μ l of PAF-AH buffer). Thirty minutes following administration of PAF-AH or buffer, mice in groups 1 and 2 were

-45-

exposed to aerosolized PBS as described below, while mice in groups 3 and 4 were exposed to aerosolized OVA. Twenty-four hours later mice were treated a second time with either 140 μ l of buffer (groups 1 and 3) or 750 units of PAF-AH in 140 μ l of buffer (groups 2 and 4) given by intravenous injection.

5 Eosinophil infiltration of the trachea was induced in the sensitized mice by exposing the animals to aerosolized OVA. Sensitized mice were placed in 50 ml conical centrifuge tubes (Corning) and forced to breath aerosolized OVA (50 mg/ml) dissolved in 0.9% saline for 20 minutes using a nebulizer (Model 646, DeVilbiss Corp., Somerset, PA). Control mice were treated in a similar manner with the
10 exception that 0.9% saline was used in the nebulizer. Forty-eight hours following the exposure to aerosolized OVA or saline, mice were sacrificed and the tracheas were excised. Tracheas from each group were inbeded in OCT and stored at -70° until sections were cut.

15 To evaluate eosinophil infiltration of the trachea, tissue sections from the four groups of mice were stained with either Luna solution and hematoxylin-eosin solution or with peroxidase. Twelve 6 μ m thick sections were cut from each group of mice and numbered accordingly. Odd numbered sections were stained with Luna stain as follows. Sections were fixed in formal-alcohol for 5 minutes at room temperature, rinsed across three changes of tap water for 2 minutes at room temperature then rinsed in two changed of dH₂O for 1 minute at room temperature. Tissue sections were stained with Luna stain 5 minutes at room temperature (Luna stain consisting of 90 ml Weigert's Iron hematoxylin and 10 ml of 1% Biebrich Scarlet). Stained slides were dipped in 1% acid alcohol six times, rinsed in tap water for 1 minute at room temperature, dipped in 0.5% lithium carbonate solution five times and rinsed in running tap water for 2 minutes at room temperature. Slides were dehydrated across 70%-95%-100% ethanol 1 minute each, at room temperature, then cleared in two changes of xylene for 1 minute at room temperature and mounted in Cytoseal 60.

20 For the peroxidase stain, even numbered sections were fixed in 4°C acetone for 10 minutes and allowed to air dry. Two hundred μ l of DAB solution was added to each section and allowed to sit 5 minutes at room temperature. Slides were rinsed in tap water for 5 minutes at room temperature and 2 drops of 1% osmic acid

-46-

was applied to each section for 3-5 seconds. Slides were rinsed in tap water for 5 minutes at room temperature and counterstained with Mayers hematoxylin at 25°C at room temperature. Slides were then rinsed in running tap water for 5 minutes and dehydrated across 70%-95%-100% ethanol 1 minute each at room temperature.
5 Slides were cleared through two changes of xylene for 1 minute each at room temperature and mounted in Cytoseal 60.

The number of eosinophils in the submucosal tissue of the trachea was evaluated. Trachea from mice from groups 1 and 2 were found to have very few eosinophils scattered throughout the submucosal tissue. As expected tracheas from
10 mice in group 3, which were pretreated with buffer and exposed to nebulized OVA, were found to have large numbers of eosinophils throughout the submucosal tissue. In contrast, the tracheas from mice in group 4, which were pretreated with PAF-AH and exposed to nebulized OVA were found to have very few eosinophils in the submucosal tissue comparable to what was seen in the two control groups, groups 1
15 and 2.

Thus, therapeutic treatment with PAF-AH of subjects exhibiting a late phase immune response involving the accumulation of eosinophils in the airway, such as that which occurs in asthma, rhinitis, and eczema, is indicated.

Example 17

20 Nearly four percent of the Japanese population has low or undetectable levels of PAF-AH activity in their plasma. This deficiency has been correlated with severe respiratory symptoms in asthmatic children [Miwa *et al.*, *J. Clin. Invest.*, 82: 1983-1991 (1988)] who appear to have inherited the deficiency in an autosomal recessive manner.

25 To determine if the deficiency arises from an inactive but present enzyme or from an inability to synthesize PAF-AH, plasma from multiple patients deficient in PAF-AH activity was assayed both for PAF-AH activity (by the method described in Example 10 for transfecants) and for the presence of PAF-AH using the monoclonal antibodies 90G11D and 90F2D (Example 13) in a sandwich ELISA as follows. Immulon 4 flat bottom plates (Dynatech, Chantilly, VA) were coated with
30 100 ng/well of monoclonal antibody 90G11D and stored overnight. The plates were

-47-

blocked for 1 hour at room temperature with 0.5% fish skin gelatin (Sigma) diluted in CMF-PBS and then washed three times. Patient plasma was diluted in PBS containing 15mM CHAPS and added to each well of the plates (50 μ l/well). The plates were incubated for 1 hour at room temperature and washed four times. Fifty μ l of 5 μ g/ml monoclonal antibody 90F2D, which was biotinylated by standard methods and diluted in PBST, was added to each well, and the plates were incubated for 1 hour at room temperature and then washed three times. Fifty μ l of ExtraAvidin (Sigma) diluted 1/1000 in CMF-PBST was subsequently added to each well and plates were incubated for 1 hour at room temperature before development.

A direct correlation between PAF-AH activity and enzyme levels was observed. An absence of activity in a patient's serum was reflected by an absence of detectable enzyme. Similarly, plasma samples with half the normal activity contained half the normal levels of PAF-AH. These observations suggested that the deficiency of PAF-AH activity was due to an inability to synthesize the enzyme or due to an inactive enzyme which the monoclonal antibodies did not recognize.

Further experiments revealed that the deficiency was due to a genetic lesion in the human plasma PAF-AH gene. Genomic DNA from PAF-AH deficient individuals was isolated and used as template for PCR reactions with PAF-AH gene specific primers. Each of the coding sequence exons were initially amplified and sequenced from one individual. A single nucleotide change within exon 9 was observed (a G to T at position 996 of SEQ ID NO: 7). The nucleotide change results in an amino acid substitution of a phenylalanine for a valine at position 279 of the PAF-AH sequence (V279F). Exon 9 was amplified from genomic DNA from an additional eleven PAF-AH deficient individuals who were found to have the same point mutation.

To test whether this mutation crippled the enzyme, an *E. coli* expression construct containing the mutation was generated by methods similar to that described in Example 10. When introduced into *E. coli*, the expression construct generated no PAF-AH activity while a control construct lacking the mutation was fully active. This amino acid substitution presumably results in a structural modification which causes the observed deficiency of activity and lack of immunoreactivity with the PAF-AH antibodies of the invention.

-48-

PAF-AH specific antibodies of the invention may thus be used in diagnostic methods to detect abnormal levels of PAF-AH in serum (normal levels are about 1 to 5 U/ml) and to follow the progression of treatment of pathological conditions with PAF-AH. Moreover, identification of a genetic lesion in the PAF-AH gene allows for genetic screening for the PAF-AH deficiency exhibited by the Japanese patients. The mutation causes the gain of a restriction endonuclease site (Mae II) and thus allows for the simple method of Restriction Fragment Length Polymorphism (RFLP) analysis to differentiate between active and mutant alleles. See Lewin, pp. 136-141 in *Genes V*, Oxford University Press, New York, New York (1994).

Screening of genomic DNA from twelve PAF-AH deficient patients was carried out by digestion of the DNA with MaeII, Southern blotting, and hybridization with an exon 9 probe (nucleotides 1-396 of SEQ ID NO: 17). All patients were found to have RFLPs consistent with the mutant allele.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.

-49-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: ICOS Corporation
- (ii) TITLE OF INVENTION: Platelet-Activating Factor Acetyl Hydrolase
- (iii) NUMBER OF SEQUENCES: 26
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Marshall, O'Toole, Gertstein, Murray & Borun
 - (B) STREET: 6300 Sears Tower, 233 South Wacker Drive
 - (C) CITY: Chicago
 - (D) STATE: Illinois
 - (E) COUNTRY: USA
 - (F) ZIP: 60606
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/133,803
 - (B) FILING DATE: 6-OCT-1993
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Noland, Greta E.
 - (B) REGISTRATION NUMBER: 35,302
 - (C) REFERENCE/DOCKET NUMBER: 32205
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (312) 474-6300
 - (B) TELEFAX: (312) 474-0448
 - (C) TELEX: 25-3658

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala
1 5 10 15

Phe

-50-

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Gln Val Leu Met Ala Ala Ala Ser Phe Gly Gln Thr Lys Ile Pro
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Lys Pro Leu Val Val Phe Val Leu Gly Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: Modified-site
(B) LOCATION: group(13, 21, 27)
(C) OTHER INFORMATION: /note= "The nucleotide at each of
these positions is an inosine."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACATGAATTG CGGNATCYTTG NGTYYTGNCCR AA

32

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-51-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TATTTCTAGA AGTGTGGTGG AACTCGCTGG

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGATGAAATTG AGCTTGAGC AGCCATCACT AC

32

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1520 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 162..1484

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCTGGTCGGA GGCTCGCAGT GCTGTCGGCG AGAACGAGTC GGGTTGGAG CGCTTGGTC

60

GCGTTGGTGC GCGGTGGAAC GCGCCCAAGGG ACCCCAGTTC CGCGGAGCAG CTCCGCGCCG

120

CGCCTGAGAG ACTAAGCTGA AACTGCTGCT CAGCTCCCAA G ATG GTG CCA CCC
Met Val Pro Pro
1AAA TTG CAT GTG CTT TTC TGC CTC TGC GGC TGC CTG GCT GTG GTT TAT
Lys Leu His Val Leu Phe Cys Leu Cys Gly Cys Leu Ala Val Val Tyr
5 10 15 20

221

CCT TTT GAC TGG CAA TAC ATA AAT CCT GTT GCC CAT ATG AAA TCA TCA
Pro Phe Asp Thr Gln Tyr Ile Asn Pro Val Ala His Met Lys Ser Ser
25 30 35

269

GCA TGG GTC AAC AAA ATA CAA GTA CTG ATG GCT GCT GCA AGC TTT GGC
Ala Trp Val Asn Lys Ile Gln Val Leu Met Ala Ala Ala Ser Phe Gly
40 45 50

317

CAA ACT AAA ATC CCC CGG GGA AAT GGG CCT TAT TCC GTT GGT TGT ACA
Gln Thr Lys Ile Pro Arg Gly Asn Gly Pro Tyr Ser Val Gly Cys Thr
55 60 65

365

-52-

GAC TTA ATG TTT GAT CAC ACT AAT AAC GGC ACC TTC TTG CGT TTA TAT Asp Leu Met Phe Asp His Thr Asn Lys Gly Thr Phe Leu Arg Leu Tyr 70 75 80	413
TAT CCA TCC CAA GAT AAT GAT CGC CTT GAC ACC CTT TGG ATC CCA AAT Tyr Pro Ser Gln Asp Asn Asp Arg Leu Asp Thr Leu Trp Ile Pro Asn 85 90 95 100	461
AAA GAA TAT TTT TGG GGT CTT AGC AAA TTT CTT GGA ACA CAC TGG CTT Lys Glu Tyr Phe Trp Gly Leu Ser Lys Phe Leu Gly Thr His Trp Leu 105 110 115	509
ATG GGC AAC ATT TTG AGG TTA CTC TTT GGT TCA ATG ACA ACT CCT GCA Met Gly Asn Ile Leu Arg Leu Leu Phe Gly Ser Met Thr Thr Pro Ala 120 125 130	557
AAC TGG AAT TCC CCT CTG AGG CCT GGT GAA AAA TAT CCA CTT GTT GTT Asn Trp Asn Ser Pro Leu Arg Pro Gly Glu Lys Tyr Pro Leu Val Val 135 140 145	605
TTT TCT CAT GGT CTT GGG GCA TTC AGG ACA CTT TAT TCT GCT ATT GGC Phe Ser His Gly Leu Gly Ala Phe Arg Thr Leu Tyr Ser Ala Ile Gly 150 155 160	653
ATT GAC CTG GCA TCT CAT GGG TTT ATA GTT GCT GCT GTA GAA CAC AGA Ile Asp Leu Ala Ser His Gly Phe Ile Val Ala Ala Val Glu His Arg 165 170 175 180	701
GAT AGA TCT GCA TCT GCA ACT TAC TAT TTC RAG GAC CAA TCT GCT GCA Asp Arg Ser Ala Ser Ala Thr Tyr Tyr Phe Lys Asp Gln Ser Ala Ala 185 190 195	749
GAA ATA GGG GAC AAG TCT TGG CTC TAC CTT AGA ACC CTG AAA CAA GAG Glu Ile Gly Asp Lys Ser Trp Leu Tyr Leu Arg Thr Leu Lys Gln Glu 200 205 210	797
GAG GAG ACA CAT ATA CGA AAT GAG CAG GCA CGG CAA AGA GCA AAA GAA Glu Glu Thr His Ile Arg Asn Glu Gln Val Arg Gln Arg Ala Lys Glu 215 220 225	845
TGT TCC CAA GCT CTC AGT CTG ATT CTT GAC ATT GAT CAT GGA AAG CCA Cys Ser Gln Ala Leu Ser Leu Ile Leu Asp Ile Asp His Gly Lys Pro 230 235 240	893
GTG AAG AAT GCA TTA GAT TTA AAG TTT GAT ATG GAA CAA CTG AAG GAC Val Lys Asn Ala Leu Asp Leu Lys Phe Asp Met Glu Gln Leu Lys Asp 245 250 255 260	941
TCT ATT GAT AGG GAA AAA ATA GCA GTC ATT GGA CAT TCT TTT GGT GGA Ser Ile Asp Arg Glu Lys Ile Ala Val Ile Gly His Ser Phe Gly Gly 265 270 275	989
GCA ACG GTT ATT CAG ACT CTT AGT GAA GAT CAG AGA TTC AGA TGT GGT Ala Thr Val Ile Gln Thr Leu Ser Glu Asp Gln Arg Phe Arg Cys Gly 280 285 290	1037
ATT GCC CTG GAT GCA TGG ATG TTT CCA CTG GGT GAT GAA GTC TAT TCC Ile Ala Leu Asp Ala Trp Met Phe Pro Leu Gly Asp Glu Val Tyr Ser 295 300 305	1085
AGA ATT CCT CAG CCC CTC TTT ATT ATC AAC TCT GAA TAT TTC CAA TAT Arg Ile Pro Gln Pro Leu Phe Ile Asn Ser Glu Tyr Phe Gln Tyr 310 315 320	1133
CCT GCT AAT ATC ATA AAA ATG AAA AAA TGC TAC TCA CCT GAT AAA GAA Pro Ala Asn Ile Ile Lys Met Lys Lys Cys Tyr Ser Pro Asp Lys Glu 325 330 335 340	1181

-53-

AGA AAG ATG ATT ACA ATC AGG GGT TCA GTC CAC CAG AAT TTT GCT GAC Arg Lys Met Ile Thr Ile Arg Gly Ser Val His Gln Asn Phe Ala Asp 345 350 355	1229
TTC ACT TTT GCA ACT GGC AAA ATA ATT GGA CAC ATG CTC AAA TTA AAG Phe Thr Phe Ala Thr Gly Lys Ile Ile Gly His Met Leu Lys Leu Lys 360 365 370	1277
GGA GAC ATA GAT TCA AAT GTA GCT ATT GAT CTT AGC AAC AAA GCT TCA Gly Asp Ile Asp Ser Asn Val Ala Ile Asp Leu Ser Asn Lys Ala Ser 375 380 385	1325
TTA GCA TTC TTA CAA AAG CAT TTA GGA CTT CAT AAA GAT TTT GAT CAG Leu Ala Phe Leu Gln Lys His Leu Gly Leu His Lys Asp Phe Asp Gln 390 395 400	1373
TGG GAC TGC TTG ATT GAA GGA GAT GAT GAG AAT CTT ATT CCA GGG ACC Trp Asp Cys Leu Ile Glu Gly Asp Asp Glu Asn Leu Ile Pro Gly Thr 405 410 415 420	1421
AAC ATT AAC ACA ACC AAT CAA CAC ATC ATG TTA CAG AAC TCT TCA GGA Asn Ile Asn Thr Thr Asn Gln His Ile Met Leu Gln Asn Ser Ser Gly 425 430 435	1469
ATA GAG AAA TAC AAT TAGGATTAAA ATAGGTTTTT TAAAAAAA AAAAAA Ile Glu Lys Tyr Asn 440	1520

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 441 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Val Pro Pro Lys Leu His Val Leu Phe Cys Leu Cys Gly Cys Leu 1 5 10 15
Ala Val Val Tyr Pro Phe Asp Trp Gln Tyr Ile Asn Pro Val Ala His 20 25 30
Met Lys Ser Ser Ala Trp Val Asn Lys Ile Gln Val Leu Met Ala Ala 35 40 45
Ala Ser Phe Gly Gln Thr Lys Ile Pro Arg Gly Asn Gly Pro Tyr Ser 50 55 60
Val Gly Cys Thr Asp Leu Met Phe Asp His Thr Asn Lys Gly Thr Phe 65 70 75 80
Leu Arg Leu Tyr Tyr Pro Ser Gln Asp Asn Arg Leu Asp Thr Leu 85 90 95
Trp Ile Pro Asn Lys Glu Tyr Phe Trp Gly Leu Ser Lys Phe Leu Gly 100 105 110
Thr His Trp Leu Met Gly Asn Ile Leu Arg Leu Leu Phe Gly Ser Met 115 120 125
Thr Thr Pro Ala Asn Trp Asn Ser Pro Leu Arg Pro Gly Glu Lys Tyr 130 135 140

-54-

Pro	Leu	Val	Val	Phe	Ser	His	Gly	Leu	Gly	Ala	Phe	Arg	Thr	Leu	Tyr
145				150				155						160	
Ser	Ala	Ile	Gly	Ile	Asp	Leu	Ala	Ser	His	Gly	Phe	Ile	Val	Ala	Ala
				165				170						175	
Val	Glu	His	Arg	Asp	Arg	Ser	Ala	Ser	Ala	Thr	Tyr	Tyr	Phe	Lys	Asp
				180				185						190	
Gln	Ser	Ala	Ala	Glu	Ile	Gly	Asp	Lys	Ser	Trp	Leu	Tyr	Leu	Arg	Thr
				195				200					205		
Leu	Lys	Gln	Glu	Glu	Glu	Thr	His	Ile	Arg	Asn	Glu	Gln	Val	Arg	Gln
				210				215					220		
Arg	Ala	Lys	Glu	Cys	Ser	Gln	Ala	Leu	Ser	Leu	Ile	Leu	Asp	Ile	Asp
				225				230			235			240	
His	Gly	Lys	Pro	Val	Lys	Asn	Ala	Leu	Asp	Leu	Lys	Phe	Asp	Met	Glu
				245				250					255		
Gln	Leu	Lys	Asp	Ser	Ile	Asp	Arg	Glu	Lys	Ile	Ala	Val	Ile	Gly	His
				260				265					270		
Ser	Phe	Gly	Gly	Ala	Thr	Val	Ile	Gln	Thr	Leu	Ser	Glu	Asp	Gln	Arg
				275				280				285			
Phe	Arg	Cys	Gly	Ile	Ala	Leu	Asp	Ala	Trp	Met	Phe	Pro	Leu	Gly	Asp
				290				295			300				
Glu	Val	Tyr	Ser	Arg	Ile	Pro	Gln	Pro	Leu	Phe	Phe	Ile	Asn	Ser	Glu
				305				310			315			320	
Tyr	Phe	Gln	Tyr	Pro	Ala	Asn	Ile	Ile	Lys	Met	Lys	Lys	Cys	Tyr	Ser
				325				330					335		
Pro	Asp	Lys	Glu	Arg	Lys	Met	Ile	Thr	Ile	Arg	Gly	Ser	Val	His	Gln
				340				345			350			350	
Asn	Phe	Ala	Asp	Phe	Thr	Phe	Ala	Thr	Gly	Lys	Ile	Ile	Gly	His	Met
				355				360			365				
Leu	Lys	Leu	Lys	Gly	Asp	Ile	Asp	Ser	Asn	Val	Ala	Ile	Asp	Leu	Ser
				370				375			380				
Asn	Lys	Ala	Ser	Leu	Ala	Phe	Leu	Gln	Lys	His	Leu	Gly	Leu	His	Lys
				385				390			395			400	
Asp	Phe	Asp	Gln	Trp	Asp	Cys	Leu	Ile	Glu	Gly	Asp	Asp	Glu	Asn	Leu
				405				410			415				
Ile	Pro	Gly	Thr	Asn	Ile	Asn	Thr	Thr	Asn	Gln	His	Ile	Met	Leu	Gln
				420				425			430				
Asn	Ser	Ser	Gly	Ile	Glu	Lys	Tyr	Asn							
				435				440							

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 504 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-55-

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 185..311

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCGCGCTCTA	GTAGAGCCGG	GCCACACAGC	CTCCCTCCCC	TACCTCCTCC	AGCATCACCG	60
AGGGGAAGGA	GAGGGTCGGG	CCACAAGGCG	CGCTAGGCAG	ACCCAGGACA	CAGCCCGCGC	120
GCAGCCCCACC	CGCCCCGGCCG	CCTGCCAGGA	GCTGCTGCGG	CCGCGCAGCC	AGGGGGACAG	180
GGGGGCTGGT	CGGAGGCTCG	CAGTGTGTC	GGCGAGAACG	AGTCGGTTT	GGAGCGCTTG	240
GGTCGCGTTG	GTGCGCGGTG	GAACGCGCCC	AGGGACCCCCA	GTTCCCGCGA	GCAGCTCCGC	300
GCGCGCCCTG	AGTGAAGGAGG	GGCCCCGGGG	GCGAGGGCGG	AGTGGGAGGA	AGGGCACCGT	360
CGCCGCGCTG	GAGGTCGGGA	CCCCGGAGCG	CGACCGGCCG	GGGTGGGCTC	GCTGAGTCG	420
ACCCGCTCTG	CTGGCCGGAC	CTGGGCTCAC	AGTCCTGCA	CCCTCGAAA	CAGCGCTAGG	480
ATCCTTCGGG	AGAGGAGAGA	TGAC				504

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 145..287

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTACCAATCT	AAAACCCAGC	ACAGAAAAAT	ACATGTTTTA	TTTTTTCCAA	GTGTTACTAG	60
TACCTCAGCC	TTCTCTGATT	TGTCAGCTTA	TTAAGGCCT	CTTCATTGCA	TACTTCTTT	120
TTCTTTTAAT	CATCTGCTTC	GAAGGGAGCT	AAGCTGAAAC	TGCTGCTCAG	CTCCCAAGAT	180
GGTGCCACCC	AAATTGCGATG	TGCTTTCTG	CCTCTGCCGC	TGCCTGGCTG	TGGTTTATCC	240
TTTTGACTGG	CAATACATAA	ATCCTGTTGC	CCATATGAAA	TCATCAGGTA	AGAGGTGTAT	300
TTGTTCAAGG	TCTTGAGCAA	CTGATCTGTC	GCCATACTTC	AACTGGGCC	CAAGAAGTTG	360
CACATCTGCA	CATCTAAACA	AGTCCTATT	AAAGGCTTAT	GGAGATCCTG	TATTCTC	417

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 498 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-56-

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 251..372

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CATAGGAGG	TAACAGTCCA	AGGCAGCTGA	GAGAAAGGCT	ATGTCTACTT	TCATCTCTTT	60
ACCCCTAAA	ACCCCTACAC	AGTGTTCAA	ACAGAGAGAC	CCTCAATAAT	TGCATATCTT	120
ACTTGTAGG	TTGAGAAAAGA	AAGAAGGCCA	GAAACTATGG	GAAGTAACTT	GATTCCGTTG	180
GAATCTTTT	GCATAATAAA	ATCTGATATG	TAATGGATGA	CAAATGAGAT	AATATTTACC	240
TGTTTTCAG	CATGGTCAA	CAAATACAA	GTACTGATGG	CTGCTGCAAC	GTTGGCCAA	300
ACTAAAATCC	CCCGGGGAAA	TGGGCCTTAT	TCCGTTGGT	GTACAGACTT	AATGTTTGAT	360
CACACTAATA	AGGTAAATGCT	TTGATTATA	CAACTTATCC	TGATACTCTA	ATATTGCTG	420
TCGCTATGGA	CCACTAGAAG	GTGTTCAAAT	GTGACCTTGC	CCTCACCTGA	GAATGACTCA	480
TTTCCATT	TGTATTGT					498

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 433 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 130..274

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGCAGCCTA	AGTCTTACA	CTTTGTGAC	ACAGAGGTAT	TGAGTCCCAC	TAATTAATAT	60
CGAAAATAGC	TGCTGGAATA	TGTTTGAGAC	ACAACCTCTC	AAAGTGC	TTAATTTCTT	120
TCTTAAACAGG	GCACCTCTT	GGCTTTATAT	TATCCATCCC	AAAGATAATGA	TCACCTTGC	180
ACCCTTGGA	TCCCAAATAA	AAAGATATTTT	TGGGGTCTTA	GCAAATTCT	TGGAACACAC	240
TGGCTTATGG	GCAACATTTC	GAGGTACTC	TTGGTAAAGA	TTCTGTTGA	TCCTTCTTTG	300
TAGGCTCTTG	CATGTATGAA	AACCTGAAA	ACAACAAGAA	CTTCAAGTAG	TTAACGACCAA	360
AGTAGATTTT	TCTTCAGTCC	AAATAGCTCC	AAAAATGATA	AGGAAAGTAT	TTCTTTAAAG	420
CCCAGGCAAC	TAC					433

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 486 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-57-

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 164..257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTGGTGGGTA TCTAGTAGCA GTCTTTAA TGAATCTACT ATTCACTCAT	AAAAAAGTAG	60
ATATAAATCA GATGGGCTCG CATTATGTC TAATGAGATA TGAATTAAT	TCACTACCAA	120
CACTCAGAGA AAACCTAAC TATAACCTTC CATTGTGTC TAGGTTCAAT	GACRACTCC	180
GCAAACTGGA ATTCCCCCTCT GAGGCCCTGGT GAAAATATC CACTTGTGT	TTTTCTCAT	240
GGTCTTGGG CATTCAAGTA ATGTTGAGA GGTTGAACAA TTTGGCTTC	CAGGAATAAA	300
TGACAAATT TTATTCAGA AAAGAAATAG CAGAGTTGG AATGTCATGC	AGGCCCTGT	360
CTGGAGGAGT TGGGGTCTCT CAATAATTGG CTGTGGTCT ATTGATCAGT	CCTAGACCTG	420
CTGGTCAAG TAGTTTTTC CCTACTATCA GCTCATTGGG ATTAGCCTCA	CAGCAGAGAA	480
GAAAGC		486

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 363 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 113..181

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCCCAGGCTC TACTACAGGG TGTAATGCC TCCATGTCC CAGTTTATT	AGTGACTCAG	60
CCTTGTAAATT CATGACTGGT AGTTGTAATT CTTCCCTCTT TTTGTTTGA	AGGACACTTT	120
ATTCTGCTAT TGGCATTGAC CTGGCATCTC ATGGGTTTAT AGTTGCTGCT	GTAGAACACAA	180
GGTATGTTAC CTGATATAAT TGGGCTCTT GGCCAACTAC AGGGAATGTC	AATGCTCATA	240
ACTATGTTTC TAATTTCTAT AAAAGTTTAT TTAAATGTT GATGGAACCTT	TCAACTATGG	300
TAACATCATG AGCAAAAGAG GAGATTGAGT TTTATCGACT TAAAGACATT	AAAAGCACCT	360
AAC		363

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 441 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

-58-

(iii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 68..191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAAC	TGAGAA	ACATGGTCAG	ATGAGGAGG	GAAGGGCAT	GCATAAATAA	TTTGCTTGT	60
ATT	ATAGA	GAGA	TAGATCTGCA	TCTGCAACT	ACTATTTCAA	GGACCAATCT	120
TAG	GGG	ACAA	TCTGGCTC	TACCTTAGAA	CCCTGAAACA	AGAGGAGGAG	180
GAA	ATGAGCA	GGTACATTG	AGTGAAGAG	GAGGTGGTTG	GTGACCTAAA	AGCATGTACA	240
AAGGGATGAC	ATTGTTAAT	TTAATTTCAC	ACCTGGCAAG	TTATGCTCCT	AGCTCTCCTA	300	
TTTCCCATT	CCTAAAGATC	TGTCATAGA	TTCCCTGGAGC	AGTAAATTC	CCTTAATGGA	360	
ATATCTAGTT	CATAGTAAA	ACAAAGGCAA	ATACAAAAT	TTGGGAGATG	ACAGTGAATA	420	
TTCAGAAATT	CTCGAGCCGG	G				441	

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 577 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 245..358

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGTTAAGTAA	ATCGTCTGAA	GTCACATAGT	AGGTAAGGCC	AAACAGAGCC	AGGATTTGGA	60
CTT	AGGCTAT	ACCTATGTG	AAAGCTGGGG	CCTGTGTCAT	TATGGTAGCA	120
ACT	AAATCAGA	TTTCCAGTTT	ATRACTGACC	AAAGATTTTT	CCCAATATACA	180
AAAC	TTAA	ATAAGTGTAA	TAACTTTTA	CTTTGTCATT	TCCTCTTCT	240
TTAGG	TACCG	CAAAGAGCAA	AAGAATGTT	CCAAGCTCTC	AGTCTGATTC	300
TCATGG	AAAG	CCAGTGAAGA	ATGCATTAGA	TTAAAGTTT	TGACATTGA	360
AAGCT	TATAAA	AAAGTAA	TTTCTCTG	TACAGTTCTT	TATTGTTTT	420
TTTC	CTGCT	ATATTGCAAG	GTACAATATG	ATAAAGGGCT	GCAACCACCC	480
GCGC	CACAC	AGACACACAA	ACCACTACAG	GTAAAGTATT	GCAGCAATGA	540
ATCTT	GGACT	AGATATGAAT	GCAGGTTAG	TCAGTTT		577

-59-

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 396 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 108..199

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATCAATGTTAT TTACCATCCC CATGAAATGA ACAATTATAT GATTGACAAA TCATTTCTTC	60
TAACACCCAG AAATAGCTAT AAATTATATAT CATGCTTTT CAAATAGGAC TCTATTGATA	120
GGGAAAAAAAT AGCAGTAAATT GGACATTCTT TTGGTGAGC AACGGTTATT CAGACTCTTA	180
GTGAAGATCA GAGATTCAAGG TAAGAAAATA AGATAGTAAAG CCAAGAGAAAT AGTAAATTAT	240
TGGAAGAAAT TATATTGTGA GATAATAATT TTATTCAAAT TCTTAGTGAA GGAACGGGAT	300
CTCTGGAGT TTATAAAGGCT ATTCTTTGTC CCCCATAAAAA TACTCTATAT ACATTTCCCT	360
AGGCTAAAAC ATCTCCCTC CTGCTATTAA AATCTC	396

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 519 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION: 181..351

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTTACAAAGT TAATCATATC CCTTTCCCCAT ATTGAAGTAT GATACCTCTT TATTCCAATC	60
AGATAACCCA TAATAAACCTG GTATGGTGC TGTCACCCAA TCCTAGCATT ATTAGGATGT	120
CCTCAATGTT GGCTAGTATG TAACCAGTTT AATTTCATCA TTGTCACACAA ATATCTACAG	180
ATGTGGTATT GCCCTGGATG CATGGATGTT TCCACTGGGT GATGAAGTAT ATTCCAGAAT	240
TCCTCAGCCC CTCTTTTTA TCAACTCTGA ATATTTCCAA TATCCTGCTA ATATCATAAA	300
AATGAAAAAA TGCTACTCAC CTGATAAAGA AAGAAAGATG ATTACAATCA GGTAAGTATT	360
AGTGACTTAT TTCATTATGT GAAACAACT TGAAGCTTGG GTAAATATCA ATCGATATCA	420
TTGGTAACT ATTAAAGAAT TGCTGAATTG GTTGTAGA CTTCAATAA GGAGAGAATT	480
AGATAATCTC AGTTTCTAAG TACATTTAGT CTACTCTT	519

-60-

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 569 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 156..304

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGAAACACAT CTAAGTAGAT CAAATTACAA	60
AGACCAACAA GACCACTTAC TTTCTTACA CTCTAACATA	120
AAATGTGAC TTTAAATGT CTTGTTCTCT TTTAGGGTT	180
GACTTCACCT TTGCAACTGG CAAAATAATT GGACACATGC	240
GATTCAATG TAGCTATTGA TCTTAGCACAC AAAGCTTCAT	300
TTAGGTAAGA AACTATTTT TTCATGACCT AAACCGAGAT	360
TCTATCTTAA TACAGCTTTA GTACTATTAA AACTATTTCC	420
AAGCAGTATA TCAATTGAA AACAGAAATT TGAGAAAGTC	480
CTATATCATA GAAAGCAAAT CAACTGTAA AGGTAAATATT	540
CTCATGTGAG GATATCGAAC GACGGTGCT	569

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 469 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 137..253

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GATACAGAGG CACATCGTCT CTACCACCT AACGGAACCT GTGTAATTG TAAATCTTA	60
TTGCCACCTA GGGGCATCCA AACTGTTAA TGCTCTCAAA AGTTAAATAT GTTGATTAAC	120
ACTTTATATT TTATAGGACT TCATAAAGAT TTGATCAGT GGGACTGCTT GATTGAAGGA	180
GATGATGAGA ATCTTATTC AGGGACCAAC ATTACACAA CCAATCAACA CATCATGTTA	240
CAGAACTCTT CAGGAATAGA GAAATACAAT TAGGATTAAC ATAGGTTTT TAAAAGTCTT	300
GTTCACAAAC TGTCTAAAAT TATGTGTGTG TGTGTGTG TGTGTGTG AGAGAGAGAG	360

-61-

AGAGAGAGAG AGAGAGAATT TTAATGTATT TTCCCAAAGG ACTCATATTT TAAAATGTAG	420
GCTATACTGT AATCGTGATT GAAGCTTGGAA CTAAGAATTT TTTCCCTTT	469

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1494 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 117..1436

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGCACGAGCT AGGATCTGAC TCGCTCTGGT GGCAATTGCTG CGCTCAGGGT TCTGGGTATC	60
CGGGAGTCAG TGCAGTGACC AGAACATCAA ACTGAAGCCA CTGCTCAGCT CCTAAG	116
ATG GTA CCA CTC AAA CTG CAG GCG CTT TTC TGC CTC CTC TGC TGC CTC Met Val Pro Leu Lys Leu Gln Ala Leu Phe Cys Leu Leu Cys Cys Leu 1 5 10 15	164
CCA TGG GTC CAT CCT TTT CRC TGG CAA GAC ACA TCT TCT TTT GAC TTC Pro Trp Val His Pro Phe His Trp Gln Asp Thr Ser Ser Phe Asp Phe 20 25 30	212
AGG CCG TCA GTA ATG TTT CAC AAG CTC CAA TCG GTG ATG TCT TCT GCT GCC Arg Pro Ser Val Met Phe His Lys Leu Gln Ser Val Met Ser Ala Ala 35 40 45	260
GGC TCT GGC CAT AGT AAA ATC CCC AAA GGA AAT GGA TCG TAC CCC GTC Gly Ser Gly His Ser Lys Ile Pro Lys Gly Asn Gly Ser Tyr Pro Val 50 55 60	308
GGT TGT ACA GAT CTG ATG TTC GGT TAT GGG AAT GAG AGC GTC TTC GTG Gly Cys Thr Asp Leu Met Phe Gly Tyr Gly Asn Glu Ser Val Phe Val 65 70 75 80	356
CGT TTG TAC TAC CCA GCT CAA GAT CAA GGT CCC CTC GAC ACT GTT TGG Arg Leu Tyr Tyr Pro Ala Gln Asp Gln Gly Arg Leu Asp Thr Val Trp 85 90 95	404
ATC CCA AAC AAA GAA TAT TTT TTG GGT CTT AGT ATA TTT CTT GGA ACA Ile Pro Asn Lys Glu Tyr Phe Leu Gly Leu Ser Ile Phe Leu Gly Thr 100 105 110	452
CCC AGT ATT GTA GGC AAT ATT TTA CAC CTC TTA TAT GGT TCT CTG ACA Pro Ser Ile Val Gly Asn Ile Leu His Leu Leu Tyr Gly Ser Leu Thr 115 120 125	500
ACT CCT GCA AGC TGG AAT TCT CCT TTA AGG ACT GGA GAA AAA TAC CCG Thr Pro Ala Ser Trp Asn Ser Pro Leu Arg Thr Gly Glu Lys Tyr Pro 130 135 140	548
CTC ATT GTC TTT TCT CAT GGT CTC GGA GCC TTC AGG ACG ATT TAT TCT Leu Ile Val Phe Ser His Gly Leu Gly Ala Phe Arg Thr Ile Tyr Ser 145 150 155 160	596

-62-

GCT ATT GGC ATT GGC TTG GCA TCT AAT GGG TTT ATA CTG GCC ACT GTC Ala Ile Gly Ile Gly Leu Ala Ser Asn Gly Phe Ile Val Ala Thr Val 165 170 175	644
GAA CAC AGA GAC AGA TCT GCA TCG GCA ACT TAC TTT TTT GAA GAC CAG Glu His Arg Asp Arg Ser Ala Ser Ala Thr Tyr Phe Phe Glu Asp Gln 180 185 190	692
GTG GCT GCA AAA GTG GAA AAC AGG TCT TGG CTT TAC CTG AGA AAA GTA Val Ala Ala Lys Val Glu Asn Arg Ser Trp Leu Tyr Leu Arg Lys Val 195 200 205	740
AAA CAA GAG GAG TCG GAA AGT GTC CGG AAA GAA CAG GTT CAG CAA AGA Lys Gln Glu Glu Ser Glu Ser Val Arg Lys Glu Gln Val Gln Gln Arg 210 215 220	788
GCA ATA GAA TGT TCC CGG GCT CTC AGT GCG ATT CTT GAC ATT GAA CAT Ala Ile Glu Cys Ser Arg Ala Leu Ser Ala Ile Leu Asp Ile Glu His 225 230 235 240	836
GGA GAC CCA AAA GAG AAT GTA CTA GGT TCA GCT TTT GAC ATG AAA CAG Gly Asp Pro Lys Glu Asn Val Leu Gly Ser Ala Phe Asp Met Lys Gln 245 250 255	884
CTG AAG GAT GCT ATT GAT GAG ACT AAA ATA GCT TTG ATG GGA CAT TCT Leu Lys Asp Ala Ile Asp Glu Thr Lys Ile Ala Leu Met Gly His Ser 260 265 270	932
TTT GGA GGA GCA ACA GTT CTT CAA GCC CTT AGT GAG GAC CAG AGA TTC Phe Gly Gly Ala Thr Val Leu Gln Ala Leu Ser Glu Asp Gln Arg Phe 275 280 285	980
AGA TGT GGA GTT GCT CTT GAT CCA TCG ATG TAT CCG GTG AAC GAA GAG Arg Cys Gly Val Ala Leu Asp Pro Trp Met Tyr Pro Val Asn Glu Glu 290 295 300	1028
CTG TAC TCC AGA ACC CTC CAG CCT CTC CTC TTT ATC AAC TCT GCC AAA Leu Tyr Ser Arg Thr Leu Gln Pro Leu Leu Phe Ile Asn Ser Ala Lys 305 310 315 320	1076
TTC CAG ACT CCA AAG GAC ATC GCA AAA ATG AAA AAG TTC TAC CAG CCT Phe Gln Thr Pro Lys Asp Ile Ala Lys Met Lys Lys Phe Tyr Gln Pro 325 330 335	1124
GAC AAG GAA AGG AAA AAT GAT TAC AAT CAA GGG CTC AGG CAC CAG AAC Asp Lys Glu Arg Lys Asn Asp Tyr Asn Gln Gly Leu Arg His Gln Asn 340 345 350	1172
TTT GAC GAC TTT ACT TTT GTA ACT GGC AAA ATA ATT GGA AAC AAG CTG Phe Asp Asp Phe Thr Phe Val Thr Gly Lys Ile Ile Gly Asn Lys Leu 355 360 365	1220
ACA CTG AAA GGA GAA ATC GAT TCC AGA GTC GCC ATC GAC CTC ACC AAC Thr Leu Lys Gly Glu Ile Asp Ser Arg Val Ala Ile Asp Leu Thr Asn 370 375 380	1268
AAA GCT TCG ATG GCT TTC TTA CAA AAG CAT TTA GGG CTT CAG AAA GAC Lys Ala Ser Met Ala Phe Leu Gln Lys His Leu Gly Leu Gln Lys Asp 385 390 395 400	1316
TTT GAT CAG TGG GAC CCT CTG GTG GAA GGA GAT GAT GAG AAC CTG ATT Phe Asp Gln Trp Asp Pro Leu Val Glu Gly Asp Asp Glu Asn Leu Ile 405 410 415	1364
CCT GGG TCA CCC TTT GAC GCA GTC ACC CAG GCC CCG CCT CAG CAA CAC Pro Gly Ser Pro Phe Asp Ala Val Thr Gln Ala Pro Ala Gln Gln His 420 425 430	1412

-63-

TCT CCA GGA TCA CAG ACC CAG RAA TAGAAGAACT TGCTTGTAC ACAGTTGCCT 1466
 Ser Pro Gly Ser Gln Thr Gln Asn
 435 440

TTTAAAAGTA GAGTGACATG AGAGAGAG 1494

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2191 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 92..1423

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCCGCGCGCTC CGGGCGGGGG ACCCTGGTTC CGCGGAGCGG CTCAGCGCGG CGCCCGGAAG 60
 TTTAACGCTGA AACCACTGCT CAGCTTCRAA G ATG TTG CCA CCC AAA CTG CAT 112
 Met Leu Pro Pro Lys Leu His
 1 5

GCG CTT TTC TGC CTC TGC AGC TGC CTC ACA CTG GTT CAT CCT ATT GAC 160
 Ala Leu Phe Cys Leu Cys Ser Cys Leu Thr Leu Val His Pro Ile Asp
 10 15 20

TGG CAA GAC CTA AAT CCT GTT GCC CAT ATT AGA TCA TCA GCA TGG GCC 208
 Trp Gln Asp Leu Asn Pro Val Ala His Ile Arg Ser Ser Ala Trp Ala
 25 30 35

AAT AAA ATA CAA GCT CTG ATG GCT GCA AGT ATT AGG CAA AGT AGA 256
 Asn Lys Ile Gln Ala Leu Met Ala Ala Ser Ile Arg Gln Ser Arg
 40 45 50 55

ATT CCC AAA GGA AAT GGA TCT TAT TCT GTC GGT TGT ACA GAT TTG ATG 304
 Ile Pro Lys Gly Asn Gly Ser Tyr Ser Val Gly Cys Thr Asp Leu Met
 60 65 70

TTT GAT TAT ACT AAT AAG GGC ACC TTT TTG CGT TTG TAT TAT CCA TCG 352
 Phe Asp Tyr Thr Asn Lys Gly Thr Phe Leu Arg Leu Tyr Pro Ser
 75 80 85

CAA GAG GAT GAC CAC TCT GAC ACG CTT TGG ATC CCA AAC AAA GAA TAT 400
 Gln Glu Asp Asp His Ser Asp Thr Leu Trp Ile Pro Asn Lys Glu Tyr
 90 95 100

TTT TTT GGT CTT AGT AAA TAT CTT GGA ACA CCC TGG CTT ATG GGC AAA 448
 Phe Phe Gly Leu Ser Lys Tyr Leu Gly Thr Pro Trp Leu Met Gly Lys
 105 110 115

ATA TTG AGC TTC TTT GGT TCA GTG ACA ACT CCT GCG AAC TGG AAT 496
 Ile Leu Ser Phe Phe Gly Ser Val Thr Thr Pro Ala Asn Trp Asn
 120 125 130 135

TCC CCT CTG AGG ACT GGT GAA AAA TAT CCA CTG ATT GTT TTT TCT CAT 544
 Ser Pro Leu Arg Thr Gly Glu Lys Tyr Pro Leu Ile Val Phe Ser His
 140 145 150

-64-

GGT CTT GGA GCA TTC CGG ACA ATT TAT TCT GCT ATT GGC ATT GAT CTA Gly Leu Gly Ala Phe Arg Thr Ile Tyr Ser Ala Ile Gly Ile Asp Leu 155	160	165	592	
GCA TCA CAT GGG TTC ATC GTC GCT GCT ATA GAA CAC AGA GAT GGA TCC Ala Ser His Gly Phe Ile Val Ala Ala Ile Glu His Arg Asp Gly Ser 170	175	180	640	
GCC TCT GCG ACT TAC TAT TTC AAG GAC CAG TCT GCT GCA GAA ATA GGG Ala Ser Ala Thr Tyr Tyr Phe Lys Asp Gln Ser Ala Ala Glu Ile Gly 185	190	195	688	
AAC AAA TCT TGG TCT TAT CTT CAA GAA CTA AAA CCA GGG GAT GAG GAG Asn Lys Ser Trp Ser Tyr Leu Gln Glu Leu Lys Pro Gly Asp Glu Glu 200	205	210	215	736
ATA CAT GTT CGA AAT GAG CAG GTA CAG AAA AGG GCA AAG GAG TGC TCC Ile His Val Arg Asn Glu Gln Val Gln Lys Arg Ala Lys Glu Cys Ser 220	225	230	784	
CAA GCT CTC AAC TTG ATT CTG GAC ATT GAT CAT GGA AGG CCA ATT AAG Gln Ala Leu Asn Leu Ile Leu Asp Ile Asp His Gly Arg Pro Ile Lys 235	240	245	832	
AAT GTC CTA GAC TTA GAG TTT GAT GTG GAA CAA CTG AAG GAC TCT ATT Asn Val Leu Asp Leu Glu Phe Asp Val Glu Gln Leu Lys Asp Ser Ile 250	255	260	880	
GAC AGG GAT AAA ATA GCA GTC ATT GGA CAT TCT TTT GGT GGA GCC ACA Asp Arg Asp Lys Ile Ala Val Ile Gly His Ser Phe Gly Gly Ala Thr 265	270	275	928	
GTT CTT CAG GCT CTT AGT GAA GAC CAG AGA TTT AGG TGC GGG ATT GCC Val Leu Gln Ala Leu Ser Glu Asp Gln Arg Phe Arg Cys Gly Ile Ala 280	285	290	295	976
TTG GAT GCA TGG ATG CTT CCA CTG GAT GAT GCA ATA TAT TCC AGA ATC Leu Asp Ala Trp Met Leu Pro Leu Asp Asp Ala Ile Tyr Ser Arg Ile 300	305	310	1024	
CCT CAG CCC CTC TTT TTT ATT AAC TCG GAA CGG TTC CAA TTT CCT GAG Pro Gln Pro Leu Phe Phe Ile Asn Ser Glu Arg Phe Gln Phe Pro Glu 315	320	325	1072	
AAT ATC AAA AAA ATG AAA AAA TGC TAC TCA CCT GAC AAA GAA AGA AAA Asn Ile Lys Lys Met Lys Lys Cys Tyr Ser Pro Asp Lys Glu Arg Lys 330	335	340	1120	
ATG ATT ACA ATC AGG GGT TCA GTC CAT CAG AAC ATT GCT GAT TTC ACT Met Ile Thr Ile Arg Gly Ser Val His Gln Asn Phe Ala Asp Phe Thr 345	350	355	1168	
TTT ACA ACT GGC AAA ATA GTT GGA TAC ATA TTC ACA TTA AAA GGA GAT Phe Thr Thr Gly Lys Ile Val Gly Tyr Ile Phe Thr Leu Lys Gly Asp 360	365	370	375	1216
ATA GAT TCA AAT GTC GCA ATT GAT CTT TGC AAC AAA GCT TCA TTG GCA Ile Asp Ser Asn Val Ala Ile Asp Leu Cys Asn Lys Ala Ser Leu Ala 380	385	390	1264	
TTT TTA CAA AAG CAT TTA GGA CTG CGG AAA GAT TTT GAT CAG TGG GAT Phe Leu Gln Lys His Leu Gly Leu Arg Lys Asp Phe Asp Gln Trp Asp 395	400	405	1312	
TCT TTG ATT GAA GGA AAA GAC GAA ATT CTT ATG CCA GGG ACC AAC ATT Ser Leu Ile Glu Gly Lys Asp Glu Asn Leu Met Pro Gly Thr Asn Ile 410	415	420	1360	

-65-

AAC ATC ACC AAC GAA CAT GAC ACT CTA CAG AAC TCT CCA GAA GCA GAG Asn Ile Thr Asn Glu His Asp Thr Leu Gln Asn Ser Pro Glu Ala Glu 425	430	435	1408
AAA TCG AAT TTA GAT TAAAAGCACT TTTTAAAGA TCTTGTAA AAACGTCAA Lys Ser Asn Leu Asp 440			1463
AAAATGTGTG TATGACTTTT AATATATTTT CTCAAATAAC TCATATTGGA AAATGTAGGC TATCCCATAA AAGTGATTGA AGCTTGGACT AGGAGGTTTT TTTCTTAA GAAAGATTGG TGTCTATCGA AATCATGCCA GCCTAAATTT TAATTTACT AAAATGATGC TGTGTCAAA TTAATTAACTA CTTTACATT CTTTAATGGA CAAGTATAAC AGGCACAAAGG CTAATGAAAA CGTGTGCAA TGACATAACA ATCCCTAAAA ATACAGATGT TCTTGCCCTCT TTTTCTATT ATAATTGAGT TTAGCAACA TGTTATGCTA GCTAGAATTT GGAAGCACTT CCCTTGACT TTGGTCATG ATAAGAAAAA TTAGATCAAG CAAATGATAA AAGCAGTGT TTACCAAGGA TTAGGGATAC TGAACAATTTC CACTATGGTA ACTGAAATGGG GAGTGACCAA GGGTAAAAAT ATTAAGGCCA AGGCAAAAGGC AGCAGATTAG AATGGATTAA AGAGAGTTA TAATTTGTTT GCATTACTT GATGGTTTAT CTCATGGATT CATGACTCAA GAAAGTGCG TAGGACACGG CAGGGATTCC AGTTATAACA CATTATTCAC CCAAAGGGTT CTTTAATTCT GTATGAGTAT TGGGAGTGGA TTAGCACATA AGAGGCATAT GTTGCTTTAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAA			1523 1583 1643 1703 1763 1823 1883 1943 2003 2063 2123 2183 2191

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 517 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 2..514

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

G GGG CAT TCT TTT GGA GGA GCA ACA GTT TTT CAA GCC CTA AGT GAA Gly His Ser Phe Gly Gly Ala Thr Val Phe Gln Ala Leu Ser Glu 1 5 10 15	46
GAC CAG AGA TTC AGA TGT GGG ATT GCC CTT GAT CCG TGG ATG TTT CCC Asp Gln Arg Phe Arg Cys Gly Ile Ala Leu Asp Pro Trp Met Phe Pro 20 25 30	94
GTG AGT GAG GAG CTG TAC TCC AGA GTT CCT CAG CCT CTC TTC TTT ATC Val Ser Glu Glu Leu Tyr Ser Arg Val Pro Gln Pro Leu Phe Phe Ile 35 40 45	142
AAC TCT GCC GAA TTC CAG ACT CCA AAG GAC ATT GCA AAA ATG AAA AAC Asn Ser Ala Glu Phe Gln Thr Pro Lys Asp Ile Ala Lys Met Lys Asn 50 55 60	190

-66-

TTC TAC CAG CCT GAC AAG GAA AGG AAA ATG ATT ACG ATC AAG GGC TCA Phe Tyr Gln Pro Asp Lys Glu Arg Lys Met Ile Thr Ile Lys Gly Ser 65 70 75	238
GTG CAC CAG AAT TTT GCT GAC GGG ACT TTT GTC ACT GGC AAA ATA ATT Val His Gln Asn Phe Ala Asp Gly Thr Phe Val Thr Gly Lys Ile Ile 80 85 90 95	286
GGA AAC AAG CTG TCA CTG AAA GGA GAC ATA GAC TCC AGA GTT GCC ATA Gly Asn Lys Leu Ser Leu Lys Gly Asp Ile Asp Ser Arg Val Ala Ile 100 105 110	334
GAC CTC ACC AAC AAG GCT TCC TTG GCT TTC TTA CAA AAA CAT TTA GGA Asp Leu Thr Asn Lys Ala Ser Leu Ala Phe Leu Gln Lys His Leu Gly 115 120 125	382
CTT CTT AAA GAC TTT GAT CAG TGG GAC TGT CTG GTG GAG GGA GAG AAC Leu His Lys Asp Phe Asp Gln Trp Asp Cys Leu Val Glu Gly Glu Asn 130 135 140	430
GAG AAC CTC ATC CCG GGG TCA CCC TTT GAT GTA GTC ACC CAG TCC CCG Glu Asn Leu Ile Pro Gly Ser Pro Asp Val Val Thr Gln Ser Pro 145 150 155	478
GCT CTG CAG AGT TCT CCC GGA TCA CAC AAC CAG AAT TAG Ala Leu Gln Ser Ser Pro Gly Ser His Asn Gln Asn 160 165 170	517

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 580 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 1..580

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAA GTA CTG ATC GCT GCA AGC TTT GGC GAA CGT AAA ATC CCT AAG Gln Val Leu Met Ala Ala Ala Ser Phe Gly Glu Arg Lys Ile Pro Lys 1 5 10 15	48
GGA AAT GGG CCT TAT TCC GTT GGT TGT ACA GAC TTA ATG TTT GAT TAC Gly Asn Gly Pro Tyr Ser Val Gly Cys Thr Asp Leu Met Phe Asp Tyr 20 25 30	96
ACT AAA AAG GGC ACC TTC TTG CGT TTA TAT TAT CCA TCC CAA GAT GAT Thr Lys Gly Thr Phe Leu Arg Leu Tyr Tyr Pro Ser Gln Asp Asp 35 40 45	144
GAT CGC CTT GAC ACC CTT TGG ATC CCA AAT AAG GAG TAT TTT TGG GGT Asp Arg Leu Asp Thr Leu Trp Ile Pro Asn Lys Glu Tyr Phe Trp Gly 50 55 60	192
CTT AGC AAG TAT CTT CGA AAA CAC TGG CTT ATG GGC AAC ATT TTG AGT Leu Ser Lys Tyr Leu Gly Lys His Trp Leu Met Gly Asn Ile Leu Ser 65 70 75 80	240

-67-

TTA CTC TTT GGT TCA GTG ACA ACT CCT GCA AAC TGG AAT TCC CCT CTG Leu Leu Phe Gly Ser Val Thr Thr Pro Ala Asn Trp Asn Ser Pro Leu 85 90 95	288
AGG CCT GGT GAA AAA TAC CCA CTT GTT TTT TCT CAT GGT CTT GGA Arg Pro Gly Glu Lys Tyr Pro Leu Val Val Phe Ser His Gly Leu Gly 100 105 110	336
GCA TTC AGG ACA ATT TAT TCT GCT ATT GGC ATT GAC CTG GCA TCT CAT Ala Phe Arg Thr Ile Tyr Ser Ala Ile Gly Ile Asp Leu Ala Ser His 115 120 125	384
GGG TTT ATA CTT GCT GCT GTA GAA CAC AGA GAT AGA TCT GCA TCT GCA Gly Phe Ile Val Ala Ala Val Glu His Arg Asp Arg Ser Ala Ser Ala 130 135 140	432
ACT TAC TAT TTC AAG AAC CAA TCT GCT GCA GAA ATA GGG AAA AAG TCT Thr Tyr Tyr Phe Lys Asn Gln Ser Ala Ala Glu Ile Gly Lys Ser 145 150 155 160	480
TGG CTC TAC CTT AGA ACC CTG AAA GAA GAG GAG GAG ATA CAT ATA CGA Trp Leu Tyr Leu Arg Thr Leu Lys Glu Glu Glu Glu Ile His Ile Arg 165 170 175	528
AAT AAG CAG GTA CGA CAA AGA GCA AAA GAA TGT TCC CAA GCT CTC ACT Asn Lys Gln Val Arg Gln Arg Ala Lys Glu Cys Ser Gln Ala Leu Ser 180 185 190	576
CTG A Leu	580

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 41 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TATTCTAGAA TTATGATACA AGTATTAATG GCTGCTGCAA G

41

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATTGATATCC TAATTGTATT TCTCTATTCC TG

32

Applicant's or agent's file
reference number

27866/32205

International application No.

-68-

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description
on page 6, lines 15 - 19

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet

Name of depository institution

American Type Culture Collection

Address of depository institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit

30 September 1994

Accession Number

HB 11724 and HB 11725

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

"In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 23(4) EPC)."

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

EP

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only

 This sheet was received with the international application

For International Bureau use only

 This sheet was received by the International Bureau on:

Authorized officer


 Signed R. Hostad
 PCT International Division

-69-

CLAIMS

We claim:

1. A purified and isolated polynucleotide encoding plasma PAF-AH.
2. A DNA according to claim 1.
3. The DNA of claim 2 which is a cDNA or a biological replica thereof.
4. The DNA of claim 2 which has the sequence set out in SEQ ID NO: 7 and encodes human plasma PAF-AH.
5. The DNA of claim 2 which encodes the human PAF-AH amino acid sequence set out in SEQ ID NO: 8.
6. The DNA of claim 2 which encodes amino acids 42 to 441 of SEQ ID NO: 8.
7. The DNA of claim 2 which is a genomic DNA or a biological replica thereof.
8. An RNA transcript of the genomic DNA of claim 7.
9. The DNA of claim 2 which is a wholly or partially chemically synthesized DNA or a biological replica thereof.

-70-

10. A full length DNA encoding PAF-AH selected from the group consisting of:

- (a) a DNA having the sequence set out in SEQ ID NO: 7;
- (b) a DNA which hybridizes under stringent conditions to the non-coding strand of the DNA of (a); and
- (c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to the non-coding strand of DNA sequence of (a) or (b).

11. The DNA of claim 2 which has the sequence set out in SEQ ID NO: 21 and encodes murine plasma PAF-AH.

12. The DNA of claim 2 which has the sequence set out in SEQ ID NO: 22 and encodes canine plasma PAF-AH.

13. An antisense polynucleotide specific for a polynucleotide according to claim 4.

14. The DNA of claim 7 further comprising an endogenous expression control DNA sequence.

15. A DNA vector comprising a DNA according to claim 2.

16. The vector of claim 15 wherein said DNA is operatively linked to an expression control DNA sequence.

17. A host cell stably transformed or transfected with a DNA according to claim 2 in a manner allowing the expression in said host cell of PAF-AH or a variant thereof possessing an enzymatic activity or immunological property specific to PAF-AH.

-71-

18. A method for producing PAF-AH, said method comprising growing a host cell according to claim 17 in a suitable nutrient medium and isolating PAF-AH or variant thereof from said cell or the medium of its growth.

19. A purified and isolated PAF-AH polypeptide consisting essentially of the human plasma PAF-AH amino acid sequence set out in SEQ ID NO: 8.

20. A purified and isolated PAF-AH polypeptide consisting essentially of amino acids 42 to 441 of SEQ ID NO: 8.

21. A monoclonal antibody specific for PAF-AH.

22. The monoclonal antibody produced by hybridoma 90G11D (ATCC HB 11724).

23. The monoclonal antibody produced by hybridoma 90F2D (ATCC HB 11725).

24. A hybridoma cell line producing a monoclonal antibody according to claim 21.

25. The hybridoma cell line 90G11D (ATCC HB 11724).

26. The hybridoma cell line 90F2D (ATCC HB 11725).

27. A humanized antibody substance according to claim 21.

-72-

28. A method for purifying PAF-AH enzyme from plasma, said method comprising the steps of:

- (a) isolating low density lipoprotein particles;
- (b) solubilizing said low density lipoprotein particles in a buffer comprising 10mM CHAPS to generate a first PAF-AH enzyme solution;
- (c) applying said first PAF-AH enzyme solution to a DEAE anion exchange column;
- (d) washing said DEAE anion exchange column using an approximately pH 7.5 buffer comprising 1mM CHAPS;
- (e) eluting PAF-AH enzyme from said DEAE anion exchange column in fractions using approximately pH 7.5 buffers comprising a gradient of 0 to 0.5 M NaCl;
- (f) pooling fractions eluted from said DEAE anion exchange column having PAF-AH enzymatic activity;
- (g) adjusting said pooled, active fractions from said DEAE anion exchange column to 10mM CHAPS to generate a second PAF-AH enzyme solution;
- (h) applying said second PAF-AH enzyme solution to a blue dye ligand affinity column;
- (i) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising 10mM CHAPS and a chaotropic salt;
- (j) applying the eluate from said blue dye ligand affinity column to a Cu ligand affinity column;
- (k) eluting PAF-AH enzyme from said Cu ligand affinity column using a buffer comprising 10mM CHAPS and imidazole;
- (l) subjecting the eluate from said Cu ligand affinity column to SDS-PAGE; and
- (m) isolating approximately 44 kDa PAF-AH enzyme from the SDS-polyacrylamide gel.

-73-

29. The method of claim 28 wherein the buffer of step (b) is 25 mM Tris-HCl, 10mM CHAPS, pH 7.5; the buffer of step (d) is 25 mM Tris-HCl, 1mM CHAPS; the column of step (h) is a Blue Sepharose Fast Flow column; the buffer of step (i) is 25mM Tris-HCl, 10mM CHAPS, 0.5M KSCN, pH 7.5; the column of step (j) is a Cu Chelating Sepharose column; and the buffer of step (k) is 25 mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, 50mM imidazole, pH 8.0.

30. A method for purifying active PAF-AH enzyme produced by *E. coli*, said method comprising the steps of:

- (a) preparing a centrifugation supernatant from lysed *E. coli* producing PAF-AH enzyme;
- (b) applying said centrifugation supernatant to a blue dye ligand affinity column;
- (c) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising 10mM CHAPS and a chaotropic salt;
- (d) applying said eluate from said blue dye ligand affinity column to a Cu ligand affinity column; and
- (e) eluting PAF-AH enzyme from said Cu ligand affinity column using a buffer comprising 10mM CHAPS and imidazole.

31. The method of claim 30 wherein the column of step (b) is a Blue Sepharose Fast Flow column; the buffer of step (c) is 25mM Tris-HCl, 10mM CHAPS, 0.5M KSCN, pH 7.5; the column of step (d) is a Cu Chelating Sepharose column; and the buffer of step (e) is 25mM Tris-HCl, 10mM CHAPS, 0.5M NaCl, 100mM imidazole, pH 7.5.

-74-

32. A method for purifying active PAF-AH enzyme produced by *E. coli*, said method comprising the steps of:

- (a) preparing a centrifugation supernatant from lysed *E. coli* producing PAF-AH enzyme;
- (b) diluting said centrifugation supernatant in a low pH buffer comprising 10mM CHAPS;
- (c) applying said diluted centrifugation supernatant to a cation exchange column equilibrated at about pH 7.5;
- (d) eluting PAF-AH enzyme from said cation exchange column using 1M salt;
- (e) raising the pH of said eluate from said cation exchange column and adjusting the salt concentration of said eluate to about 0.5M salt;
- (f) applying said adjusted eluate from said cation exchange column to a blue dye ligand affinity column; and
- (g) eluting PAF-AH enzyme from said blue dye ligand affinity column using a buffer comprising about 2M to about 3M salt; and
- (h) dialyzing said eluate from said blue dye ligand affinity column using a buffer comprising about 0.1% Tween.

33. The method of claim 31 wherein the buffer of step (b) is 25mM MES, 10mM CHAPS, 1mM EDTA, pH 4.9; the column of step (c) is an S sepharose column equilibrated in 25mM MES, 10mM CHAPS, 1mM EDTA, 50mM NaCl, pH 5.5; PAF-AH is eluted in step (d) using 1mM NaCl; the pH of the eluate in step (e) is adjusted to pH 7.5 using 2M Tris base; the column in step (f) is a sepharose column; the buffer in step (g) is 25mM Tris, 10mM CHAPS, 3M NaCl, 1mM EDTA, pH 7.5; and the buffer in step (h) is 25mM Tris, 0.5M NaCl, 0.1% Tween 80, pH 7.5.

34. A method for detecting PAF-AH enzyme in serum comprising the step of contacting serum with PAF-AH-specific monoclonal antibody or antibodies and quantifying the amount of PAF-AH enzyme bound by said antibody or antibodies.

-75-

35. A method for treating a mammal susceptible to or suffering from a PAF-mediated pathological condition comprising administering PAF-AH to said mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF in said mammal.

36. A method for treating a mammal susceptible to or suffering from pleurisy comprising administering PAF-AH to said mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF of said mammal.

37. A method for treating a mammal susceptible to or suffering from asthma comprising administering PAF-AH to said mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF of said mammal.

38. A method for treating a mammal susceptible to or suffering from rhinitis comprising administering PAF-AH to said mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF in said mammal.

39. A method for treating a mammal susceptible to or suffering from eczema comprising administering PAF-AH to said mammal in an amount sufficient to supplement endogenous PAF-AH activity and to inactivate pathological amounts of PAF in said mammal.

40. A method for detecting a genetic lesion in the human PAF-AH gene resulting in a substitution of a phenylalanine residue for a valine residue at amino acid 279 of the human plasma PAF-AH enzyme comprising using restriction fragment length polymorphism analysis to differentiate between wild type and mutant alleles.

1 / 10

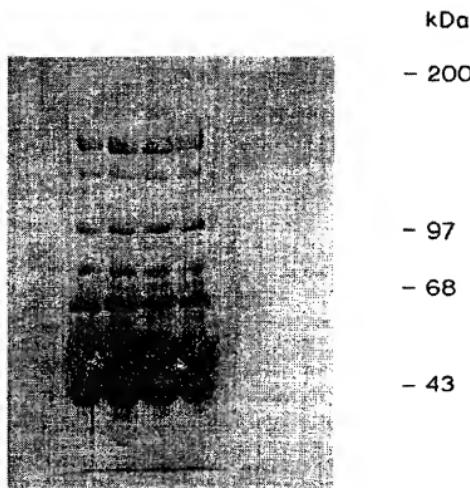


FIGURE 1

2 / 10

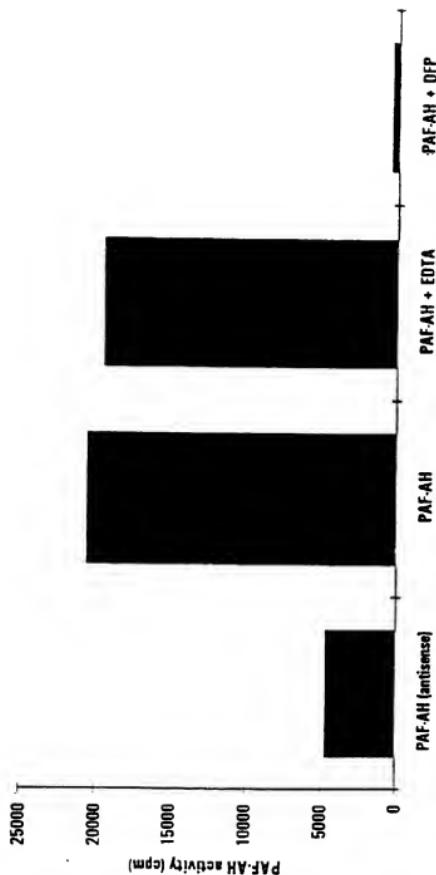
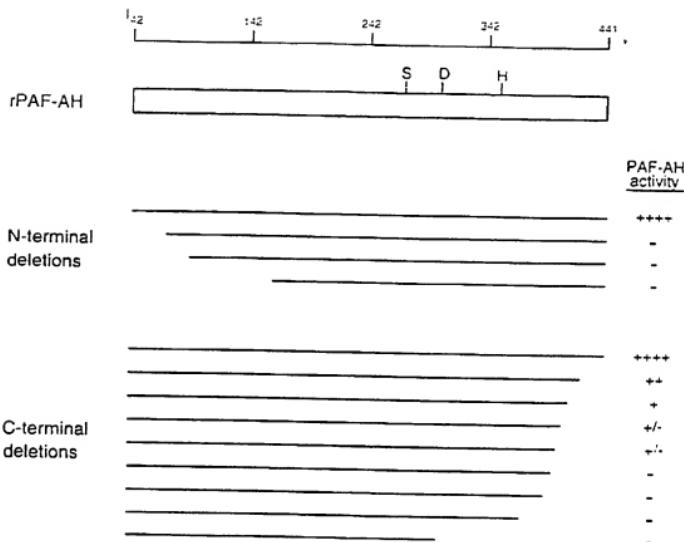
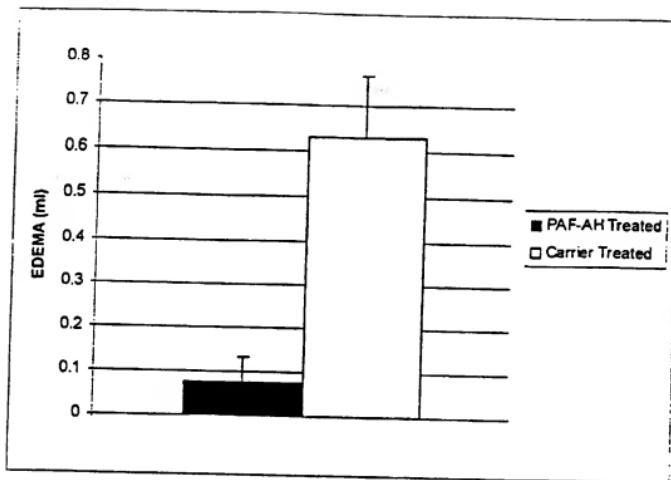


FIGURE 2

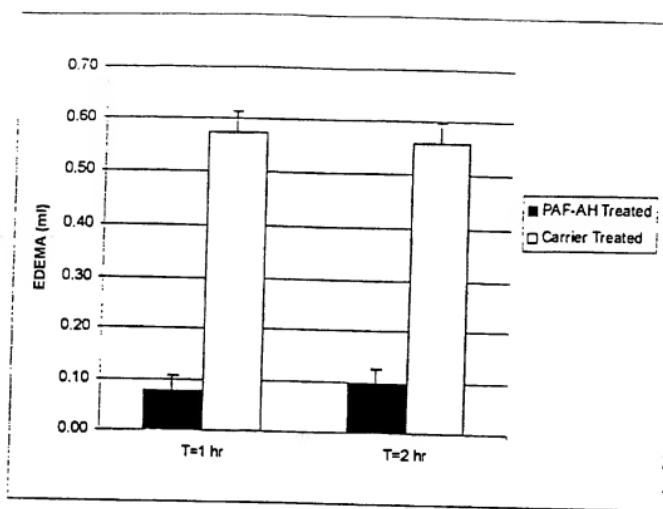
3 / 10

**FIGURE 3**

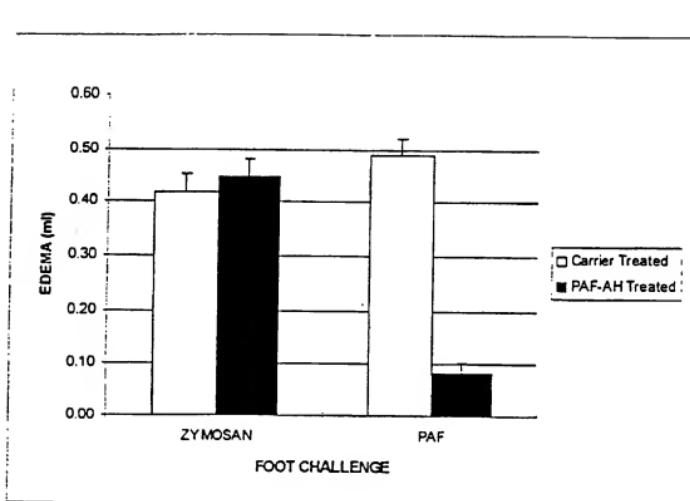
4 / 10

**FIGURE 4**

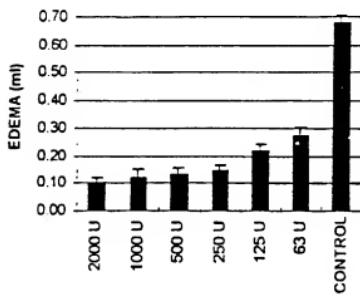
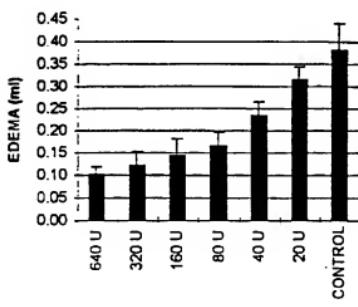
5 / 10

**FIGURE 5**

6 / 10

**FIGURE 6**

7 / 10

Experiment 1**FIGURE 7A****Experiment 2****FIGURE 7B**

8 / 10

Experiment 1

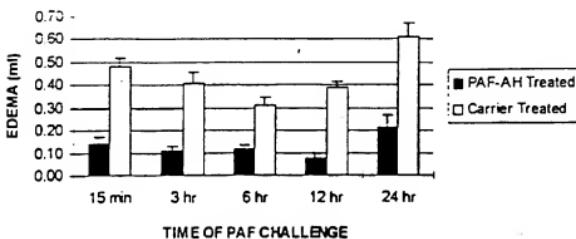


FIGURE 8A

Experiment 2

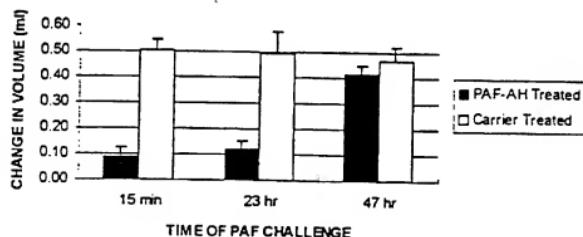
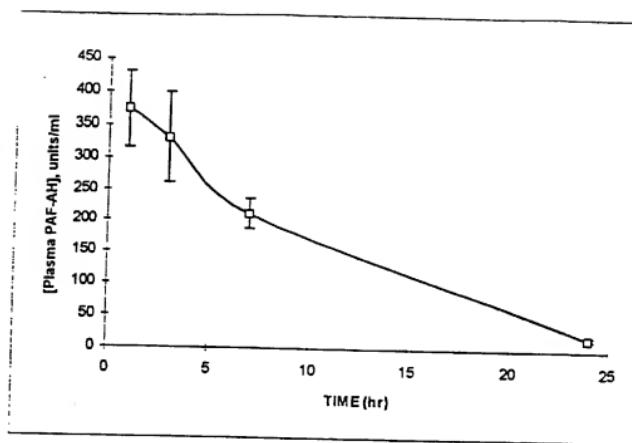


FIGURE 8B

9 / 10

**FIGURE 9****SUBSTITUTE SHEET (RULE 26)**

10 / 10

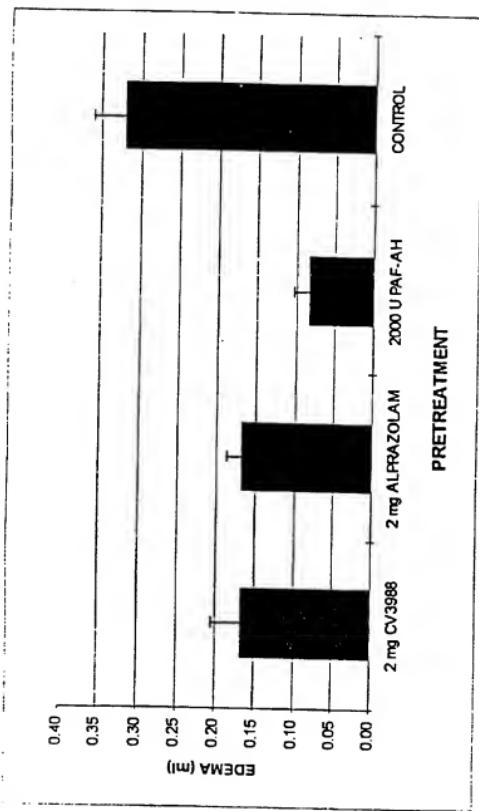


FIGURE 10

SUBSTITUTE SHEET (RULE 26)

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/55 C12N9/18 A61K38/43 C12P21/02 C07K16/40
 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N C07K A61K C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.265, no.17, June 1990, BALTIMORE, MD US pages 9682 - 9687 STAFFORINI D. M. ET AL. 'Human macrophages secrete platelet-activating factor' cited in the application see the whole document ---	19-21, 28,29, 34-39
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.262, no.9, March 1987, BALTIMORE, MD US pages 4223 - 4230 STAFFORINI D. M. ET AL. 'Human plasma platelet-activating factor acetylhydrolase' cited in the application see the whole document ---	19-21, 28,29, 34-39

-/-



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

*' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is considered in combination with one or more other such documents, such combination being obvious to a person skilled in the art.

*' document member of the same patent family

Date of the actual completion of the international search

2 January 1995

Date of mailing of the international search report

23 -01- 1995

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patendaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+ 31-70) 340-3016

Authorized officer

Espen, J

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.86, April 1989, WASHINGTON US pages 2393 - 2397 STAFFORINI M. D. ET AL. 'Lipoproteins alter the catalytic behavior of the platelet-activating factor acetylhydrolase in human plasma' see the whole document <p style="text-align: center;">---</p>	19-21, 28,29, 34-39
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.268, no.6, February 1993, BALTIMORE, MD US pages 3857 - 3865 STAFFORINI D. M. ET AL. 'The platelet-activating factor acetylhydrolase from human erythrocytes' see page 3858 <p style="text-align: center;">---</p>	21
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.269, no.37, 16 September 1994, BALTIMORE, MD US pages 23150 - 23155 HATTORI M. ET AL. 'The catalytic subunit of bovine brain platelet-activating factor acetylhydrolase is a novel type of serine esterase' cited in the application see the whole document <p style="text-align: center;">---</p>	1
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.262, no.9, March 1987, BALTIMORE, MD US pages 4215 - 4222 STAFFORINI D. M. ET AL. 'Human plasma platelet-activating factor acetylhydrolase' see the whole document <p style="text-align: center;">-----</p>	
2		